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Hyperrecombination in *Streptococcus pneumoniae* Depends on an Atypical *mutY* Homologue

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The unusual behavior of the mutation *ami36*, which generates hyperrecombination in two point crosses, was previously attributed to a localized conversion process changing A/G mispairs into CG pairs. Although the mechanism was found to be dependent on the DNA polymerase I, the specific function responsible for this correction was still unknown. Analysis of the pneumococcal genome sequence has revealed the presence of an open reading frame homologous to the gene *mutY* of *Escherichia coli*. The gene *mutY* encodes an adenine glycosylase active on A/G and A/7,8-dihydro-8-oxoguanine (8-OxoG) mismatches, inducing their repair to CG and C/8-OxoG, respectively. Here we report that disrupting the pneumococcal *mutY* homologue abolishes the hyperrecombination induced by *ami36* and leads to a mutator phenotype specifically enhancing AT-to-CG transversions. The deduced amino acid sequence of the pneumococcal MutY protein reveals the absence of four cysteines, highly conserved in the endonuclease III/MutY glycosylase family, which ligate a $[4Fe\text{-}4S]^{2+}$ cluster. The actual function of this cluster is still intriguing, inasmuch as we show that the pneumococcal gene complements a *mutY* strain of *E. coli*.

In transformation of *Streptococcus pneumoniae*, double-stranded DNA binds to the membrane and is randomly cleaved (see reference 23 for a review). Then, single-stranded segments enter the cell from a 3′ end while the complementary strands are degraded to oligonucleotides with the opposite polarity (32). About half of the entering segments integrate into the chromosome by homologous recombination (21). The recombination process is RecA dependent (37), exchanges strands from 5′ to 3′ relative to the donor (43), and forms a donor-recipient structure which is heteroduplex when the donor and the recipient sequences are not identical (11). Both strands of the donor DNA have the same probability of entering a cell so that two complementary heteroduplexes are generated in equal frequency among the recipient bacteria (7). Pneumococcal transformation therefore allows study of the in vivo processing of heteroduplexes such as base-base mismatches. In particular, the variability observed in the transformation efficiencies of point mutations led to the discovery of a mismatch repair system (10, 20, 55). This system, called Hex, recognizes the different mismatches with varying efficiencies and induces the complete excision of the donor strand (31). Base mismatches are ranked as a function of decreasing repair efficiency by Hex as follows: G/T = A/C = G/G > C/T > A/A > T/T > A/G > C/C (6). The deletions and the additions of one or two nucleotides lead to mismatches that are very efficiently recognized by Hex (13, 14). The heterologies longer than 2 bases lead to heteroduplexes which are poorly recognized by Hex, and for those longer than 5 bases, there is no repair at all by Hex (13, 22) nor by any bacterial repair or conversion system (42).

A Hex-independent repair, specific for A/G mismatches, was found in *S. pneumoniae*. The existence of such a system was signalled by the hyperrecombination—i.e., the abnormally high frequency of wild-type transformants—shown by the mutation *ami36* when involved in two point crosses (24). The mutation *ami36* results from a CG-to-AT transversion and, consequently, forms upon transformation the mismatches A^{36}/G^{+} and C^{+}/T^{36}. It was shown that only A/G triggers hyperrecombination (51), leading to an excess of wild-type but not double-mutant transformants (38). In addition, we know that this hyperrecombination requires the pneumococcal *polA* product, which is a functional homolog of the *Escherichia coli Pol*I protein, and that upon transformation, about 50% of the A^{36}/G^{+} mismatches lead to CG independently of the replication (41). These results have suggested the existence of a repair specifically changing A/G mismatches to CG pairs in *S. pneumoniae*. In contrast to Hex, this repair seems specific for A/G mismatches, changes A/G into CG irrespective of the recipient strand, and is closely localized around the mismatch (12).

Shortly after the proposal of an A/G-to-CG conversion in *S. pneumoniae*, a similar repair system was found in *E. coli*, depending on the gene *mutY* (2, 26). In vitro analyses have identified MutY as an adenine glycosylase specific for A/G mispairs (3). The complete A/G-to-CG repair requires a short patch resynthesis by the DNA polymerase I (47, 56). Further studies have suggested that the major in vivo substrate for MutY is the adenine from A/7,8-dihydro-8-oxoguanine (8-OxoG) mismatches (34). This finding indicates that MutY, as the 8-OxoG glycosylase MutM and the 8-OxodGTPase MutT, should antagonize the mutagenicity of 8-OxoG, a major product of oxidative damage of DNA (see references 17 and 35 for reviews). The gene *mutY* is partly homologous to the gene *nth* of *E. coli*, which encodes the DNA glycosylase endonuclease III (EndoIII) involved in the removal of oxidatively damaged pyrimidines (36). In particular, MutY and EndoIII display a four-cysteine domain which ligate a $[4Fe\text{-}4S]^{2+}$ cluster presumably involved in specific DNA recognition (16, 44).

Based on the conserved domains shared by MutY, EndoIII, and a third related protein (40), we designed degenerate oligonucleotides to probe by Southern and PCR analysis the pneumococcal genome to identify a *mutY* homologue. Such investigations being unsuccessful we left this question, until most of the pneumococcal genome sequence became available,

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
S. pneumoniae		
R800	R6 derivative, *ami*$^+$ *hex*$^+$	29
R801	R800 *hexB*	29
553	R801 *ami36*	Michel Sicard[a]
800T51	R800 *mutY*::pRT51	This work
801T51	R801 *mutY*::pRT51	This work
553T51	553 *mutY*::pRT51	This work
E. coli		
DH5α	*supE44* Δ*lacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
AB1157	*thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 kdgK51 proA2 his-4 argE3 str-31 tsx33 supE44*	2
AB1157-Y11	AB1157 *mutY zgd*::Tn*10*	2
Plasmids		
pR350	pSK+ derivative, Ap^r Sp^r	8
pRT51	pR350 with 229 bp internal to the pneumococcal *mutY*	This work
pAM238	pGB2 derivative allowing lacZΔM15 α-complementation, Sp^r	Kaymeuang Cam[a]
pAMY2	pAM238 with the pneumococcal *mutY* on a 1,400-bp fragment	This work
pAMΔY	pAMY2 with deletion of the 600-bp upstream region of *mutY*	This work

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revealing the presence of a putative *mutY* homologue. However, this gene lacks the conserved cysteine domain, which may account for our previous failure to identify it by DNA probing. The aim of this work was to characterize the pneumococcal *mutY* homologue, mainly to analyze its involvement in the hyperrecombination induced by *ami36* and its functional relatedness with the MutY protein of *E. coli*.

MATERIALS AND METHODS

Strains, plasmids, media, transformation procedures, and transformation efficiencies. Strains and plasmids are listed in Table 1. The pneumococcal strains were cultured at 37°C in CAT medium (46). The selection of *ami*$^+$ bacteria was carried out on synthetic medium containing excess isoleucine (50). *S. pneumoniae* was transformed as described (43). The transformation efficiency of a chromosomal marker is the number of transformants for this marker divided by the number of transformants for a reference marker also carried on the chromosome, in order to correct for fluctuations in competence. Usually the point mutation *str41* which confers resistance to streptomycin is the reference marker.

The following antibiotics and concentrations were used for the selection of *S. pneumoniae* transformants or mutants: 5 μM methotrexate, rifampin (1 μg/ml), streptomycin (200 μg/ml), and spectinomycin (200 μg/ml). *E. coli* was grown in Luria-Bertani medium (48). For the detection of *mutY*-pAM238 recombinant clones, the white-blue screen was used by adding 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) and isopropyl-β-D-thiogalactoside (IPTG) (40 μg/ml). M9 minimal medium prepared as described (48) and supplemented with 0.2% Casamino acids was used with AB1157/pAMY2 strains for induction or repression of the *lacZ* promoter. Induction was achieved by adding 2 mM IPTG and 0.4% glycerol; repression was achieved by adding 0.4% glucose. Ligation products and plasmids were introduced in *E. coli* strains by electrotransformation. The antibiotics and concentrations used for the selection of *E. coli* transformants or mutants were ampicillin (100 μg/ml), rifampin (100 μg/ml), and spectinomycin (100 μg/ml).

DNA techniques. Plasmids were extracted from *E. coli* by the alkaline lysis method (48). Chromosomal extraction of *S. pneumoniae* was performed as described (5). PCR amplifications were done with the Hot Tub DNA polymerase (Amersham). The oligonucleotides used for the amplification of the pneumococcal *rpoB* region involved in the resistance to rifampin were upstream, 5′-CGCTTCTTTGACCCACGTCG, and downstream, 5′-CCGTCAGCGATGAAATCGCC. Sequencing of the Rif^r mutations was performed directly on the PCR products, with the CircumVent Thermal Cycle DNA Sequencing Kit (New England Biolabs) and using the following internal primer: 5′-GACAATGAAGTCTTGACACC. Sequencing the cloned *mutY* gene in the region of the expected cysteines was performed on the plasmid pAMY2 using a CEQ 2000 apparatus and a CEQ dye terminator cycle sequencing kit (Beckman). The oligonucleotide used to prime this sequence was: 5′-TGCGGGTCTTGGCGCGTCTG.

Construction of strains disrupted for the *mutY* homologue. A 575-bp fragment, internal to the pneumococcal *mutY* homologue, was amplified from chromosomal DNA of the strain R800, using the following primers: upstream, 5′-TTGCCTTGGAGGAGAAGTAA, and downstream, 5′-ATTCTGATATGCCGCACTAA. The PCR product was digested with *Hin*dIII and *Hin*cII, generating an *Hin*dIII-*Hin*cII fragment of 227 bp and two flanking fragments. The 229-bp fragment was cloned in pR350 digested with *Hin*dIII and *Hin*cII. The resulting recombinant plasmid pRT51 was used to transform the *S. pneumoniae* strains R800, R801, and 553, in order to inactivate by homology-directed insertion the *mutY*-like gene (30). The transformants resistant to spectinomycin were selected.

Cloning of the pneumococcal *mutY* homologue. A DNA segment of 1,561 bp including the open reading frame (ORF) containing the *mutY* homologue was obtained by PCR amplification, using chromosomal DNA of the strain R800 as a template, and the following oligonucleotides: up, 5′-CAGCTTCACCTTGCCGTAGG, and down, 5′-TGCTCTAGCGCTTCACGACC. Further cloning is described in Fig. 1.

Homology searches, genome analysis, and multiple sequence alignments. Finding sequences sharing homology with the proteins MutY, EndoIII, and MutM of *E. coli* was performed with the TBLASTN search on microbial genomes, finished and unfinished (http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html). Finding the complete sequence of the *mutY* gene of *S. pneumoniae* was possible due to the generosity of The Institute for Genomic Research (TIGR) allowing us to access the unfinished pneumococcal genome. Alignments of the protein sequences were performed using the Multalin program (http://www.toulouse.inra.fr/multalin.html).

Nucleotide sequence accession number. The sequence of the internal region of the *mutY* gene of the pneumococcal strain R800 has been assigned EMBL accession no. AJ271596.

RESULTS

Presence of an atypical *mutY* homologue in the pneumococcal genome. The hyperrecombination induced by *ami36* in pneumococcus relies on an A/G-to-CG repair (51). Such a repair might be mediated by a protein related to MutY, the adenine glycosylase of *E. coli* which removes A from A/G mispairs, allowing their repair to CG pairs. Inasmuch as more than 90% of the pneumococcal genome sequence was available, we have looked for a *mutY*-like gene in this sequence. A BLAST search revealed the presence of a putative ORF whose deduced translation leads to a protein containing 391 amino acids and displaying 30% identity with MutY of *E. coli* (Fig. 2). This ORF lies in the contiguous region Sp80 of the pneumococcal genome sequenced by TIGR. The presence of putative promoter and terminator sequences upstream and downstream of the ORF suggests that it can be transcribed as a single gene. As shown (Fig. 2), the deduced amino acids sequence of the putative pneumococcal *mutY* gene does not display four cysteines, characteristic of the MutY/EndoIII family, and which are involved in the formation of a $[4Fe-4S]^{2+}$ cluster. We have

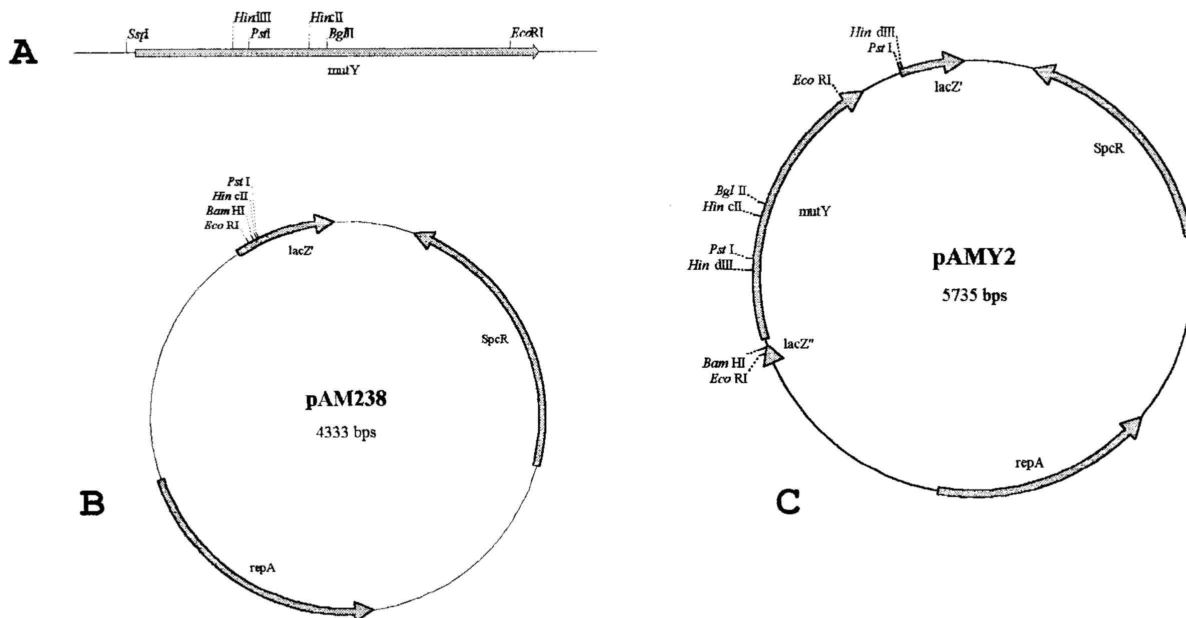

FIG. 1. The 1,561-bp fragment (A) containing the pneumococcal *mutY* was cut with *Ssp*1, leaving 17 bp before the −35 region of the putative promoter. The resulting 1,402-bp fragment was directly ligated into pAM238 (B) linearized with the blunt cutting enzyme *Hinc*II. The ligation mix was used to transform the *E. coli* strain DH5α. Spectinomycin-resistant transformants leading to white colonies on IPTG–X-gal-containing medium were cultured, and their plasmids were extracted and analyzed by restriction digests, *Eco*RI, *Pst*I, *Eco*RI/*Pst*I, and *Hin*dIII. One recombinant clone, called pAMY2 (C), displaying the 1,402-bp fragment with the *mutY* gene oriented downstream of the *lac* promoter, was kept for further analysis.

sequenced the internal region of the cloned *mutY* gene of our wild-type strain R800 and confirmed this atypical feature.

The *mutY* gene controls hyperrecombination. In crosses involving two linked *ami* mutations, one donor and one recipient,

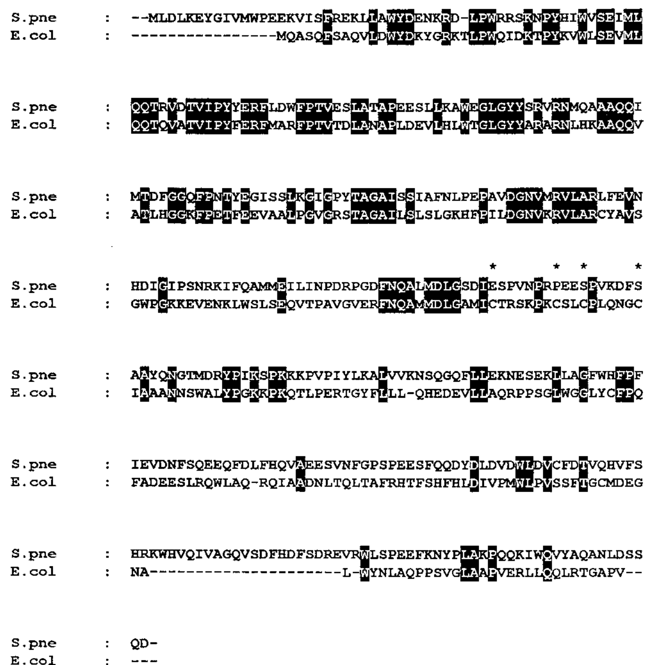

FIG. 2. Alignment of the MutY amino acid sequence of *E. coli* (E.col) (GI accession number 1789331) and the 391-amino-acid sequence deduced from a putative ORF found in the *S. pneumoniae* (S.pne) genome sequence. Shading highlights identical amino acids in both sequences. The asterisks indicate the positions of the four cysteines present in the *E. coli* protein but lacking in the ORF of *S. pneumoniae*.

breaks or recombinations occur between the two sites involved in the cross. Such breaks generate transformants which are either double *ami* mutants or ami^+. The mutations of the *ami* locus confer resistance to methotrexate, but double mutants cannot be distinguished by this procedure, while ami^+ bacteria are selectable in synthetic medium containing excess isoleucine (Ile^r) (50). The number of breaks and recombination events occurring between two closely linked sites, thus the number of Ile^r transformants, is proportional to the physical distance separating the two sites (4). With regard to this rule of proportionality, *ami36* generates an abnormally high frequency of ami^+ transformants. For example, crosses between *ami36* and *ami6*, 27 bp apart, produce 20 to 25% ami^+ bacteria while about 1% is predicted by the distance (24). The pneumococcal strains 553 (*hexB ami36* $mutY^+$) and 553T51 (*hexB ami36 mutY*::pRT51), were transformed with a chromosomal DNA carrying the mutations *ami6* and *str41* in order to test whether the hyperrecombination induced by *ami36* was affected by the inactivation of *mutY*. Hex-deficient strains were used as the recipients to avoid interferences between the Hex system and the hyperrecombination process (24). Hyperrecombination was abolished in the strain 553T51 (Table 2). The proportion of double transformants, ami^+ *str41*, which lead to bigger colonies than single *str41* transformants, was 20 times lower in the strain 553T51, confirming that hyperrecombination was suppressed.

Disruption of the pneumococcal *mutY* confers a mutator phenotype. Hyperrecombination depends on the repair to CG of an A/G mismatch formed upon transformation. If this repair is also active on the mismatches which form spontaneously within the chromosome, the mutants affected for this function might display a high mutation frequency. The mutation frequencies of resistance to streptomycin (Sm^r), rifampin (Rif^r), and methotrexate (Mtx^r) of the pneumococcal strains R800 and 800T51 were investigated (Tables 3 and 4). Single colonies were picked and used to inoculate liquid cultures that were

TABLE 2. Inactivation of *mutY* abolishes hyperrecombination

Recipient strain	Donor DNA	No. of transformants/ml		Recombination index[a]
		Ami^+	Sm^r	
553 (*ami36 hexB mutY*$^+$)	*ami6 str41*	7.9×10^5	3.8×10^6	21
553T51 (*ami36 hexB mutY*)	*ami6 str41*	3.5×10^4	3.6×10^6	1

[a] The ratio of Ami^+ to Sm^r transformants, given as a percentage.

then grown to stationary phase and plated on selective media (Table 3). Alternatively, the colonies were picked and directly streaked on solid selective media to score the mutants appeared within the colonies (Table 4). While the rate of Sm^r mutants remained unchanged (not shown), both methods indicate that the proportions of Mtx^r and Rif^r mutants were enhanced in 800T51 compared with R800. Streaking the colonies on selective media and scoring the mutants grown on the streaks appears to be an easy way to estimate accurately the mutation frequency.

The pneumococcal *mutY* gene specifically prevents CG-to-AT transversions. Mtx^r mutations, which inactivate the *ami* operon, may arise throughout within the 6,000 bp of the *ami* locus. The sequence determination of the mutations appearing in such a long locus requires at first the localization of these mutations. By contrast, the Rif^r mutations are usually located in the gene *rpoB*, which encodes the β-subunit of the RNA polymerase, and map mostly in a region of about 300 bases, called cluster I in *E. coli* (19). The determination of the DNA changes which arise in independent Rif^r mutants should reveal the mutator specificity of the strain 800T51. Searching the pneumococcal genome sequence (with the TIGR database) indicates that cluster I is almost identical in pneumococcus and other bacteria, as confirmed recently by Enright et al. (9). A 1,500-bp segment including cluster I was amplified in independent Rif^r mutants. All the amplified fragments carried the Rif^r mutation as verified by their ability to transform a Rif^s strain to the Rif^r phenotype. A third oligonucleotide located 50 bp upstream of cluster I was used to prime the sequencing reactions directly on the PCR products. Out of 23 independent Rif^r mutants, the mutations were found on three codons of cluster I, among which was the codon for serine 495 (Table 5). Rif^r mutations in this codon were previously reported in *E. coli* but not in *S. pneumoniae* (9, 19). We have detected GC-to-TA (and CG-to-AT) transversions in three types of Rif^r mutants out of five types characterized. Such transversions represent 3 mutations out of 9 sequenced in R800, and 13 mutations out of 14 sequenced in 800T51. CG-to-AT transversions are therefore specifically enhanced in the strain disrupted for the *mutY* homologue.

Transformation efficiencies of CG-to-AT transversions. Crosses between *ami36* and *ami6* produce about 20% *ami*$^+$ transformants instead of the 1% predicted on the basis of the 27 bp separating the two mutations. The correction of A^{36}/G^+ mismatches to C^+G^+ pairs by MutY is responsible for this excess of *ami*$^+$ transformants. In transformations of an *ami*$^+$ strain with *ami36* DNA, the MutY correction must occur similarly, changing the same amount of A/G mismatches to CG pairs. In a *mutY*$^+$ strain, the transformation efficiency of *ami36* should display a decrease of about 20% compared with the transformation efficiency in a *mutY* strain. If we assume that in a *hexB mutY* background, i.e., without any potential repair, the transformation efficiency of *ami36* is 1, then a 20% decrease in a *hexB mutY*$^+$ should lead to an efficiency of 0.8. Despite the normal fluctuation occurring in the measurements of the transformation efficiencies, an efficiency of 0.8 should be distinct from an efficiency of 1. We have constructed a *hexB mutY* strain by disrupting the *mutY* gene of the *hexB* strain R801, leading to the strain 801T51. Chromosomal DNA containing the mutations *ami36* and *str41* was used to transform R801 (*hexB mutY*$^+$ *ami*$^+$) and 801T51 (*hexB mutY ami*$^+$). The same transformations were carried out with chromosomal DNA containing the mutations *ami6* and *str41. ami6* is a spontaneous mutation corresponding to a GC-to-AT transition (6), which generates upon transformation the mismatches A/C and G/T. On average (Table 6) and in a reproducible way, *ami36* displays a transformation efficiency reduced by 0.2 in the *mutY*$^+$ background, while *ami6* remains mostly unaffected.

To test the same question with CG-to-AT mutations other than *ami36*, we have measured the transformation efficiencies of different Rif^r mutations previously sequenced (Table 5). The DNAs extracted from the Rif^r mutants were used to transform a strain with the mutation *str41*. The Rif^r transformants were selected, and their DNAs, carrying both a Rif^r mutation and *str41*, were used to transform the strains R801 and 801T51. The ratios of Rif^r to Sm^r transformants were measured as transformation efficiencies. On average, the differences in transformation efficiency observed between a *mutY* and a *mutY*$^+$ background were −0.05, 0.1, and 0.21 for CG-to-AT mutations type 1, 3, and 5, respectively. These values are weak and are close to 0.13, the efficiency measured for the Rif^r mutation type 4, which does not generate an A/G mismatch upon transformation. Although generating a donor-recipient A/G mismatch, these Rif^r mutations are globally not, or not strongly, affected by the MutY repair. However, we cannot exclude the possibility that Rif^r type 5 has undergone a significant repair, close to the one observed for *ami36*.

TABLE 3. Mutator phenotype of pneumococcal strain 800T51 as shown by plating liquid cultures[a]

Strain	No. of cultures with mutants/ total no. of cultures		Avg no. of mutant colonies/ 10^9 bacteria		Frequency of mutant colonies relative to R800	
	Mtx^r	Rif^r	Mtx^r	Rif^r	Mtx^r	Rif^r
R800 (*hex*$^+$ *mutY*$^+$)	10/10	22/42	4,700	16	1	1
800T51 (*hex*$^+$ *mutY*)	10/10	39/39	21,000	530	4	33

[a] Liquid cultures inoculated from single colonies were grown to stationary phase and plated on antibiotic agar plates (40 μl was plated for Mtx^r; 400 μl was plated for Rif^r).

TABLE 4. Mutator phenotype of pneumococcal strain 800T51 as shown by streaking colonies[a]

Strain	No. of streaks with mutants/total no. of streaks		Avg no. of mutant colonies/streak		Frequency of mutant colonies relative to R800	
	Mtx^r	Rif^r	Mtx^r	Rif^r	Mtx^r	Rif^r
R800 (*hex*$^+$ *mutY*$^+$)	41/57	28/420	1.2	0.069	1	1
800T51 (*hex*$^+$ *mutY*)	71/73	138/469	3.6	0.35	3	5

[a] Single colonies were picked and directly inoculated on antibiotic agar plates by a single streak.

Complementation of a *mutY* strain of *E. coli*. To investigate whether the *mutY* gene of *S. pneumoniae* could complement a *mutY* mutant of *E. coli*, we cloned it into pAM238, a low-copy-number plasmid. In the recombinant plasmid pAMY2, the pneumococcal gene, which nevertheless keeps its own putative promoter, is oriented so that transcription could also occur from the *lacZ* promoter. The control plasmid pAMΔY derives from pAMY2 by *Bam*HI-*Bgl*II digestion (Fig. 1), leading to a 599-bp deletion, including the promoter and the 545 adjacent base pairs of the pneumococcal *mutY* gene. The strains AB1157 and AB1157-Y11 containing pAMY2 or pAMΔY were grown on Luria-Bertani agar plates. Independent colonies were streaked on rifampin-containing medium to analyze the mutator phenotype (Table 7). Suppression of the *mutY* mutator phenotype was observed with pAMY2, not with pAMΔY, suggesting that the pneumococcal *mutY* gene encodes a protein functionally similar to the adenine glycosylase MutY of *E. coli*. The complementation was confirmed from liquid cultures and from colonies grown on minimal medium allowing or not allowing gene induction from the *lacZ* promoter (not shown). The repression of the *lacZ* promoter had no influence on the complementation by pAMY2, suggesting that the cloned gene is transcribed from its own promoter.

DISCUSSION

The hyperrecombination induced by the mutation *ami36* is triggered by an A/G-to-CG correction system requiring the DNA polymerase I of pneumococcus. However, the genetic control of this repair was still unknown. As the sequence flanking *ami36* looked important for this hyperrecombination (12, 24), it was actually unclear whether this pathway was more related to MutY, which repairs A/G to CG, or to the VSP pathway, which repairs G/T to GC in defined sequence environments (reviewed in reference 25). As we show here that the disruption of a pneumococcal *mutY* homologue abolishes hyperrecombination, we can assume that the main protein controlling the A/G-to-CG repair involved in this hyperrecombination is a MutY-like adenine glycosylase. We have found that *mutY* strains are mutators, suggesting that this pathway is active not only on the mismatches occurring upon transformation, but also on the spontaneous ones. The Rif^r mutation pattern reveals an enhancement of CG-to-AT transversions in the pneumococcal *mutY* background, which is expected if MutY repairs A/G mismatches to CG pairs. Also confirming the specialized action of *mutY* is the fact that no increase in the Sm^r mutant frequency was detected in a *mutY* background. Indeed, only AT-to-CG transversions in the *str* gene, which encodes the ribosomal protein S12, have been found to confer the Sm^r phenotype (33).

A comparison between the spontaneous mutation frequencies and the transformation efficiencies of the Rif^r mutations characterized in this work is relevant. In a *mutY* background, we have observed at least a fivefold increase of the frequency of Rif^r mutants (Table 4). These mutants are mostly due to CG-to-AT mutations (Table 5), and we can estimate at least a 10-fold increase of these transversions. Such an increase is observed in a *mutY*-deficient strain, suggesting that, in a *mutY*$^+$ background, at least 90% of the A/G mismatches are corrected to CG pairs if the pneumococcal MutY is specific for A/G mispairs. Transforming Rif^r bacteria with a DNA mutated to Rif^r by a CG-to-AT change generates either A/G or C/T mismatches in the recipient cells. Assuming that MutY repairs at least 90% of the A/G mismatches to CG pairs and has no effect on C/T mismatches, the transformation efficiency of such Rif^r mutations should be reduced by at least 45% in a *mutY*$^+$ strain compared with a *mutY* strain. Such a reduction was not observed. The best decrease we have detected is a 20% decrease with Rif^r type 5. The transformation efficiencies of the Rif^r mutations rather suggest that the MutY system has a weak effect on A/G mismatches created upon transformation. To render this observation compatible with the high and specific mutagenicity of *mutY* strains, we propose that the A/G mis-

TABLE 5. Sequences of the Rif^r mutations[a]

Strain and Rif type	Triplet 1465–1467 (a.a. 489)	Triplet 1483–1485 (a.a. 495)	Triplet 1465–1467 (a.a. 499)	No. of mutants
R800 (*hex*$^+$ *mutY*$^+$)				
Wild-type Rif^s	GAC (Asp)	TCT (Ser)	CAC (His)	
Rif^r type 1	TAC (Tyr)			1
Rif^r type 2		TTT (Phe)		2
Rif^r type 3			AAC (Asn)	2
Rif^r type 4			TAC (Tyr)	4
800T51 (*hex*$^+$ *mutY*)				
Rif^r type 1	TAC (Tyr)			3
Rif^r type 5		TAT (Tyr)		4
Rif^r type 3			AAC (Asn)	6
Rif^r type 4			TAC (Tyr)	1

[a] Triplets and amino acid (a.a.) numbers are relative to the pneumococcal *rpoB* gene. Asp 489, Ser 495, and His 499 correspond to Asp 516, Ser 522, and His 526, respectively, in the *E. coli rpoB* gene. Only the mutated codons are indicated in the rows of data for Rif^r types.

TABLE 6. Transformation efficiencies of *ami* and Rif[r] mutations

Cross (strain[a] × DNA[b])	Transformation efficiencies[c] measured in independent crosses[d]	Avg efficiency
R801 × *ami36*	0.62, 0.77, 0.82, 0.88, 0.88, 0.88, 0.91, 0.91, 0.92, 1.03, 1.19	0.89
801T51 × *ami36*	0.86, 0.9, 0.95, 1.03, 1.06, 1.10, 1.10, 1.13, 1.35, 1.38	1.09
R801 × *ami6*	0.73, 0.77, 1.07, 1.10, 1.10, 1.17, 1.22, 1.28	1.06
801T51 × *ami6*	0.83, 0.92, 0.96, 0.96, 0.98, 1.04, 1.1, 1.19, 1.25, 1.25	1.05
R801 × Rif[r] type 1	0.85, 0.97, 1.15	0.99
801T51 × Rif[r] type 1	0.78, 1, 1.05	0.94
R801 × Rif[r] type 3	0.91, 0.93, 1.01	0.95
801T51 × Rif[r] type 3	0.94, 1.09, 1.13	1.05
R801 × Rif[r] type 5	0.92, 0.94	0.93
801T51 × Rif[r] type 5	1.06, 1.23	1.14
R801 × Rif[r] type 4	0.81, 0.93, 1.01	0.92
801T51 × Rif[r] type 4	1.03, 1.05, 1.06	1.05

[a] R801 is *hexB mutY*$^+$ and 801T51 is *hexB mutY*.
[b] Each DNA carries the mutation *str41* in addition to the indicated *ami* or *rif* mutation.
[c] Ratios of Mtx[r] to Sm[r] transformants for *ami36* and *ami6* as donor DNAs and of Rif[r] to Sm[r] transformants for Rif[r] type 1, 3, 4, and 5 as donor DNAs.
[d] In each cross two independent platings were done for each phenotype (Mtx[r], Rif[r], or Sm[r]), to give the averaged indices indicated.

matches which form upon transformation might be structurally different from those which form spontaneously, in particular because upon transformation a three-stranded DNA could be formed.

Alternatively, the best substrate for the pneumococcal system might not be A/G but A/8-OxoG mismatches. In *E. coli*, A/8-OxoG is thought to be the primary in vivo substrate for the adenine glycosylase MutY and the major cause of CG to-AT-transversions observed in the *mutY* strains (34). As the pneumococcal wild-type gene cloned on a low-copy-number plasmid suppresses the mutator phenotype of an *E. coli mutY* mutant, we can assume that the pneumococcal function is similar to that of the *E. coli* MutY glycosylase and must be able to trigger the change A/8-OxoG to C/8-OxoG. In addition, it is well established in *E. coli* that MutY cooperates with the proteins MutT and MutM to form an antimutator system specialized against the mutagenic potential of 8-OxoG, which relies mainly on its capability to pair with adenine (53). MutT, first identified as a dGTPase, is mainly involved in the hydrolysis of oxodGTP to oxodGMP (27), and MutM is a DNA glycosylase specialized for the removal of 8-OxoG from C/8-OxoG mispairs (54). Interestingly, the pneumococcal *mutX* gene was found to be functionally similar to *mutT* (33), and searching the pneumococcal genome reveals a putative *mutM*-like gene. The presence of three genes in *S. pneumoniae*, homologous to *mutY*, *mutT*, and *mutM*, strongly suggests that the same antimutator system exists in *S. pneumoniae*. The oxidative stress and the mutagenicity it generates must be actual problems for this nonrespiratory bacterium.

The deduced amino acid sequence of the pneumococcal MutY does not display the highly conserved stretch of four cysteines, Cys-X_6-Cys-X_2-Cys-X_5-Cys, which coordinates a [4Fe-4S]$^{2+}$ cluster loop (36), called FCL. Searching the genome sequences reveals no eukaryotic MutY lacking this domain (not shown) and just two bacterial MutY proteins lacking the cysteine domain, those of *Treponema pallidum* and *Streptococcus pyogenes* (Fig. 3). The MutY of *S. pyogenes* is similar to the pneumococcal one, in particular in the region where the cysteines were expected (Fig. 3), suggesting that the FCL domain was absent before divergence of the two species. In *E. coli* this domain is reported to be crucial for the specific DNA substrate recognition by MutY (16, 44). As the pneumococcal *mutY* displays a CG-to-AT antimutator action which complements the MutY deficiency in *E. coli*, the absence of an FCL domain in the pneumococcal protein is intriguing. Structural differences in the protein of *S. pneumoniae* might compensate for the absence of an iron-sulfur cluster. The C-terminal domain of the pneumococcal protein, which is reported in *E. coli* to be more specifically involved in 8-OxoG recognition (15, 39), is more divergent than the rest of the protein (Fig. 2) and could lead to structural differences. Nevertheless, despite its antimutator effect complementing in vivo the MutY deficiency of *E. coli*, the pneumococcal glycosylase might display a weaker activity than the *E. coli* glycosylase especially toward the A/G mismatch. In *E. coli*, although A/G is not the main in vivo substrate of MutY, in vitro studies indicate that the relative glycosylase activity of MutY toward A/G is not weaker than toward A/8-Oxo-G (34), although the kinetic and the mechanism of the reaction are not identical for A/G and A/8-oxoG (45). In *S. pneumoniae*, the correction by MutY of A/G mismatches is undetected upon transformation of Rif[r] mutations types 1 and 3 (Table 6). It is detected but weak upon transformation of *ami36* and, less significantly, of Rif[r] mutation type 5 (Table 6). Previous studies have indicated that the sequence adjacent to the A/G mismatch is important for hyperrecombination and should be related to the structure generated by *ami36*: 5′ATTAAT-3′TAAGTA (12). The heteroduplex structures generated by the Rif[r] mutations are as follows, for type 1, 3, and 5, respectively: 5′CCAAGT-3′GGTGCA, 5′TCTAAC-3′AGAGTG, and 5′TTTATG-3′AAAGAC. Out of the three tested Rif[r] mutations, type 5 only might have undergone a detectable repair by MutY. Interestingly, the heteroduplex structure generated by Rif[r] mutation type 5 is quite related to the one generated by *ami36*. In particular three AT pairs are localized 5′ to the mismatched adenine in both *ami36* and Rif[r] type 5. An attractive hypothesis is that the absence of an FCL domain on the pneumococcal MutY is specifically detrimental

TABLE 7. The pneumococcal *mutY*$^+$ gene complements an *E. coli mutY* strain

E. coli strain (genotype)	Plasmid	No. of streaks with mutant colonies[a]/ total no. of streaks	Avg no. of mutants/streak
AB1157 (*mutY*$^+$)	pAMΔY	5/40	0.2
AB1157 (*mutY*$^+$)	pAMY2	5/34	0.3
AB1157Y11 (*mutY*)	pAMΔY	27/39	3
AB1157Y11 (*mutY*)	pAMY2	7/38	0.3

[a] Single colonies of the indicated strain containing the indicated plasmid were picked and inoculated on rifampin agar plates by a single streak.

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                       *      *  *     *
H.pyl   :  NHNQALIDLGALICSP-KPKCAICPFNPYCLG--KNHLERHTLKKKQEII
B.sub   :  EFNQGLMELGALICTPKSPSCLLCPVQQHCSAFEEGTERELPVKSKKKKP
H.inf   :  DFNQAMMDIGAMVCMRTKPKCDLCPLNIDCLAYKNTNWEKFPAKKPKKAM
E.col   :  RFNQAMMDLGAMICTRSKPKCSLCPLQNGCIAAANNSWALYPGKKPKQTL
N.men   :  AYTQGLMDLGATVCKRTKPLCHQCPMADICEAKKQNRTAELPRKKTAAEV
S.pne   :  DFNQALMDLGSDIESPVNPRPEESPVKDFSAAYQNGTMDRYPIKSPKKKP
S.pyo   :  DFNQALMDLGTDIESAKTPRPDESPIRFFNAAYLNGTYGKYPIKNPKKKP
M.tub   :  EFSVALMELGATVCTARTPRCGLCPLD-WCAWRHAGYPPSDGPPRRGQA-
S.coe   :  RWAAASMELGALVCTAKKESCHRCPIAAQCAWRLAGKPAHDGPPRRGQT-
T.pal   :  RWYYALMDYGAVLKRKITNPNRRSKHYVKQSPFEGSLRQVRGAVLRE---
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FIG. 3. Alignment of the cysteine-containing region in some bacterial MutY homologues. Asterisks indicate the position of the cysteines. Shading highlights amino acid identity in at least 6 sequences out of 10. Origins of the proteins and their GI accession numbers (in parentheses) are as follows: H.pyl, *Helicobacter pylori* (4154640); B.sub, *Bacillus subtilis* (2633186); H.inf, *Haemophilus influenzae* (1573768); E.col, *E. coli* (1789331); N.men, *Neisseria meningitidis* (3860539); S.pne, *S. pneumoniae*; S.pyo, *Streptococcus pyogenes*; M.tub, *Mycobacterium tuberculosis* (2950412); S.coe, *Streptomyces coelicolor* (4585587); T.pal., *T. pallidum* (3322622).

to the repair of A/G mismatches, which consequently might occur in a few sequence contexts only. Although the biological importance of A/G mismatches arising in vivo is unknown, they may represent a minor source of CG-to-AT transversions. The relatively high AT content of the pneumococcal chromosome, 60% compared with 50% in *E. coli*, might originate in part in the inefficient repair of A/G mismatches by a handicapped MutY protein.

The unusual lack of the FCL domain in the pneumococcal MutY made us wonder whether similar features are also absent from related proteins in *S. pneumoniae*. We have detected in the pneumococcal genome a putative MutM homologue and a putative EndoIII homologue. MutM is an oxoG glycosylase specific for A/8-OxoG mismatches and displays a zinc finger motif coordinated by four cysteines (54). The same structure was found in the putative MutM homologue of *S. pneumoniae*. EndoIII has a glycosylase activity specific for damaged pyrimidines and usually displays a four-cysteine domain identical to the MutY one, also ligating a $[4Fe\text{-}4S]^{2+}$ cluster. Interestingly, the putative EndoIII found in *S. pneumoniae* displays only the two first cysteines of a putative four-cysteine domain (not shown). To support this observation, homologues of the pneumococcal EndoIII protein were searched for in the microbial genomes. An amino acid sequence sharing the same half-cysteine domain was found in *Streptococcus mutans* only, a streptococcal species strongly related to *S. pneumoniae*. This observation suggests that the EndoIII of some streptococcal species has lost the carboxy-terminal part of an ancestral cysteine domain and should lack the iron-sulfur cluster. That an iron-sulfur cluster is lacking in EndoIII should not indicate that the activity is lost. Two EndoIII activities have been characterized in *Saccharomyces cerevisiae*, one of which lacks the $[4Fe\text{-}4S]^{2+}$ cluster. Both are active but display some differences in the substrate specificities (1, 49). Concerning *S. pneumoniae*, the absence of a $[4Fe\text{-}4S]^{2+}$ ligating domain in both MutY and the putative EndoIII suggests that both proteins have evolved so that the iron-sulfur cluster is not required. It is tempting to speculate that the scarcity of iron in the natural environment of *S. pneumoniae* and its importance for bacterial growth and virulence (18, 52), have partly oriented such an evolution.

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