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dim-2 encodes a DNA methyltransferase responsible for all known cytosine methylation in *Neurospora*

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To understand better the control of DNA methylation, we cloned and characterized the *dim-2* gene of *Neurospora crassa*, the only eukaryotic gene currently known in which mutations appear to eliminate DNA methylation. The *dim-2* gene is responsible for methylation in both symmetrical and asymmetrical sites. We mapped *dim-2* between *wc-1* and *un-10* on linkage group (LG) VIIR and identified the gene by RFLP mapping and genetic complementation. *Dim-2* encodes a 1454 amino acid protein including a C-terminal domain homologous to known DNA methyltransferases (MTases) and a novel N-terminal domain. Neither a deletion that removed the first 186 amino acids of the protein nor a mutation in a putative nucleotide binding site abolished function, but a single amino acid substitution in the predicted catalytic site did. Tests for repeat-induced point mutation (RIP) indicated that *dim-2* does not play a role in this process, i.e. duplicated sequences are mutated in *dim-2* strains, as usual, but the mutated sequences are not methylated, unlike the situation in *dim-2*$^+$ strains. We conclude that *dim-2* encodes an MTase that is responsible for all DNA methylation in vegetative tissues of *Neurospora.*

Keywords: *dim-2*/DNA methyltransferase/methylation mutant/*Neurospora*/repeat-induced point mutation

Introduction

DNA methylation is common in organisms as diverse as bacteria, fungi, higher plants and mammals. In eukaryotes, methylation is almost exclusively found at the C5 position of selected cytosines; and in many organisms, nearly all 5mC is found in symmetrical sites, such as the sequence 5′CpG/3′GpC. This is thought to reflect the operation of a 'maintenance DNA methyltransferase (MTase)' that preferentially recognizes and methylates hemimethylated sites (Holliday and Pugh, 1975; Riggs, 1975). Methylation patterns are consistently maintained, but it is common to find cytosines that are 'partially' methylated, i.e. methylated in some fraction of a cell population. The status of any given site presumably reflects a combination of *de novo* methylation, maintenance methylation and demethylation, e.g. by failure to methylate DNA after replication. Remarkably little is known about what determines whether a particular sequence or site is subject to these processes in eukaryotes. This has motivated us to study control of methylation in a model system, the filamentous fungus *Neurospora crassa*.

The function of DNA methylation is somewhat better understood than its control. It is known that DNA methylation can indirectly interfere with transcription initiation (Kass *et al*., 1997; Schubeler *et al*., 2000) or transcription elongation (Barry *et al*., 1993; Rountree and Selker, 1997), and methylation is involved in a range of epigenetic phenomena, such as X chromosome inactivation (Beard *et al*., 1995; Panning and Jaenisch, 1996; Heard *et al*., 1997), genomic imprinting (Li *et al*., 1993; Peterson and Sapienza, 1993; Bartolomei and Tilghman, 1997) and silencing of selfish DNA (Bestor, 1996; Selker, 1997). DNA methylation is required for normal development in both mammals and plants. Methylation deficiencies in mice homozygous for mutations in either of two MTase genes, *Dnmt1* (Li *et al*., 1992) or *Dnmt3b* (Okano *et al*., 1999), lead to lethality during embryonic development. Mutations in a third MTase known to be active in mouse, *Dnmt3a*, cause mice to become runted and to die at 4 weeks of age (Okano *et al*., 1999). In humans, mutations in *DNMT3B* are associated with an autosomal recessive disorder, ICF syndrome (Hansen *et al*., 1999; Okano *et al*., 1999; Xu *et al*., 1999). In the plant *Arabidopsis thaliana*, reduction in DNA methylation, caused by expression of a MTase (MET1) antisense construct or by a mutation in a modifier gene (*ddm1*), results in flowering abnormalities, including homeotic transformations of floral organs and partial female sterility (Finnegan, 1996; Kakutani *et al*., 1996; Ronemus *et al*., 1996). The various consequences of reduced DNA methylation in animals and plants may result from expression of genes normally silenced by methylation.

One approach to elucidating the control and function of DNA methylation is to identify and characterize the components of the methylation machinery, starting with the MTases. The first eukaryotic DNA MTase to be characterized, DNMT1, was isolated from mouse (Bestor *et al*., 1988). Although DNMT1 appears responsible for the maintenance of most DNA methylation in mammals, mutant cells that produce no DNMT1 retain a low level of methylation and are capable of *de novo* methylation (Li *et al*., 1992; Lei *et al*., 1996). Three other potential DNA MTase genes were found in mouse and human databases: *Dnmt2* (Okano *et al*., 1998; Yoder and Bestor, 1998), and *Dnmt3a* and *Dnmt3b* (Okano *et al*., 1998). Biochemical studies demonstrated that *Dnmt3a* and *Dnmt3b* are bona fide DNA MTases and genetic studies suggested that these two enzymes are responsible for *de novo* methylation in embryonic cells (Hsieh, 1999; Okano *et al*., 1999; Robertson *et al*., 1999). Sequence conservation among DNA MTases facilitated isolation of putative MTase genes

from several other model eukaryotes, including *A.thaliana* (Finnegan and Dennis, 1993; Scheidt *et al*., 1994; Henikoff and Comai, 1998; Genger *et al*., 1999), *Xenopus laevis* (Kimura *et al*., 1996), *Ascobolus immersus* (Chernov *et al*., 1997; Malagnac *et al*., 1997, 1999), *Aspergillus nidulans* (D.W.Lee, M.Freitag, E.U.Selker and R.Aramayo, personal communication), the fission yeast *Schizosaccharomyces pombe* (Wilkinson *et al*., 1995) and *Drosophila melanogaster* (Hung *et al*., 1999; Tweedie *et al*., 1999; Gowher *et al*., 2000; Lyko *et al*., 2000). Some of these organisms lack obvious DNA methylation, suggesting that their MTase-like sequences may represent pseudogenes. Some putative DNA MTase genes from organisms with obvious methylation may also be inactive or relatively insignificant. For example, disruption of the known *Ascobolus* DNA MTase-like genes (*masc1* and *masc2*), singly or together, does not noticeably affect methylation in this fungus, and attempts to detect MTase activity *in vitro* from Masc1 have failed (Malagnac *et al*., 1997).

Genetic approaches to identifying the components of the methylation machinery offer notable advantages over molecular approaches. First, no prior knowledge about the identity of the 'players' is required. Thus, a mutant hunt for genes that affect DNA methylation may uncover novel MTases as well as unexpected components of the methylation machinery. Secondly, by definition, genetic approaches reveal genes that have a recognizable phenotype. This makes it possible to distinguish between genes that are major players and genes with minor roles (and pseudogenes). To date, DNA methylation mutants have been successfully isolated in *A.thaliana* (Vongs *et al*., 1993) and the filamentous fungus *N.crassa* (Foss *et al*., 1993, 1995, 1998). In the case of *Arabidopis*, this approach yielded mutants in two genes, a previously identified DNA MTase, MET1 (E.Richards, personal communication), and *DDM1* (decrease in DNA methylation; Kakutani *et al*., 1995, 1996, 1999; Jeddeloh *et al*., 1999), a protein related to the yeast SWI/SNF2 family of chromatin-remodeling proteins. Mutant hunts in *Neurospora* have revealed a greater number of genes involved in DNA methylation—five so far—but with the exception of the gene described here, their identities are not yet known. One possible reason why genetic studies have revealed more methylation genes in *Neurospora* than in plants or animals is that DNA methylation appears to be non-essential in this organism: mutations in *dim-2* (defective in methylation) appear to eliminate all DNA methylation without causing growth defects (Foss *et al*., 1993). This is consistent with the idea that the primary function of DNA methylation in *Neurospora* is to control 'selfish' DNA. Although a sizeable fraction of the cytidines in the wild-type genome of *Neurospora* is methylated (~1.5%; Foss *et al*., 1993), all known functional genes are unmethylated. Methylation appears concentrated in relics of RIP (repeat induced point mutation), a genome defense system that operates specifically in the haploid nuclei of dikaryotic cells formed by fertilization (Selker *et al*., 1987a; Grayburn and Selker, 1989; Selker, 1990; Kinsey *et al*., 1994; Perkins *et al*., 1997; Margolin *et al*., 1998). RIP searches for linked and unlinked sequence duplications, such as those that result from transposons, and then introduces numerous G:C to A:T transitions in both copies of the duplicated sequences (Selker and Garrett, 1988; Cambareri *et al*., 1989). All duplicated sequences longer than ~1 kb are efficiently inactivated by the mutations and/or by methylation, which is typically found on remaining cytosines in sequences heavily mutated by RIP. The relationship between RIP and DNA methylation is not well understood. On the one hand, it has been shown that RIP frequently creates 'signals' for *de novo* cytosine methylation that cause the mutated sequences, and some surrounding sequences, to be methylated in vegetative tissues (Selker *et al*., 1993; Singer *et al*., 1995b; Irelan and Selker, 1997; Miao *et al*., 2000). On the other hand, it has been suspected that methylation of cytosines may also be a part of the RIP mutagenic mechanism in the sexual dikaryotic cells (Selker, 1990). Consistent with this possibility, an analogous process called MIP (methylation induced premeiotically), which operates in the fungus *A.immersus*, results in methylation of cytosines without concomitant mutations in duplicated sequences (Rhounim *et al*., 1992; Rossignol and Faugeron, 1994). Interestingly, inactivation of the *Ascobolus* DNA MTase-like gene *masc1* interferes with MIP, but, as noted above, does not appear to affect DNA methylation in vegetative tissues (Malagnac *et al*., 1997).

As a step towards a better understanding of the control of DNA methylation in eukaryotes, we cloned and characterized the *dim-2* gene of *N.crassa*, currently the only gene known in which mutations appear to abolish all DNA methylation. Loss of all methylation is particularly striking in *Neurospora* because this organism normally has 5mC in both symmetrical and asymmetrical sites (Selker and Stevens, 1985; Selker *et al*., 1993). We wished to determine whether *dim-2* encodes a key regulator of the methylation machinery and/or encodes a novel methyltransferase. We also addressed the role of Dim-2 in the RIP process. We report that *dim-2* encodes a novel MTase responsible for all DNA methylation that has been detected in *N.crassa*, and that the *dim-2* MTase plays no role in mutagenesis by RIP.

Results

Previous work has shown that the original mutation in *dim-2* eliminated methylation and allowed transcription of genes that were methylated after the action of RIP (Foss *et al*., 1993; Rountree and Selker, 1997). Nevertheless, neither the original *dim-2* mutation identified in a heavy chemical mutagenesis (Foss *et al*., 1993), nor an allele identified in a subsequent screen for spontaneous mutants (J.Irelan, A.Tharp, P.Paine and E.U.Selker, unpublished), display a strong phenotype that would allow for selection of transformants complementing the mutation. We therefore decided to clone the *dim-2* gene by positional cloning using cosmid, lambda and YAC libraries and to test candidate clones for complementation of the methylation defect of a *dim-2* strain. As the first step, we built *dim-2*/*dim-2*$^+$ heterokaryons to verify that the original mutation could be complemented (Figure 1). To test for induction of methylation in *dim-2* nuclei, the *mtr*^{RIP8} allele, which is methylated in *dim-2*$^+$ strains (Figure 1B, lanes 1 and 2), was included in the *dim-2* partner, and an *mtr* deletion was included in the *dim-2*$^+$ partner (Figure 1A). Heterokaryons with various ratios of *dim-2*$^+$/*dim-2* nuclei (1:1 to 1:200) were constructed and tested for methylation of *mtr*^{RIP8} by

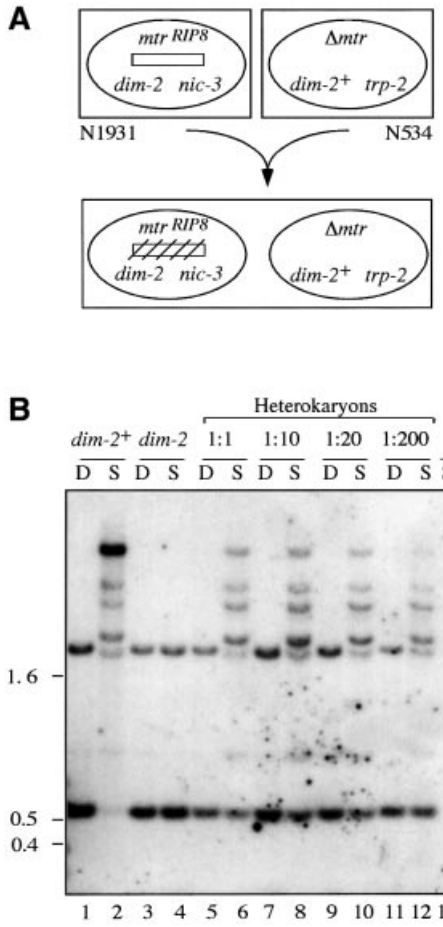

Fig. 1. The *dim-2*^{1} mutation is complemented in heterokaryons. (**A**) Schematic diagram of the experiment. Two compatible auxotrophic *N.crassa* strains (N1931 and N534) were forced to form a heterokaryotic strain on minimal medium. The initially unmethylated *mtr*^{RIP8} allele (open rectangle) of the *dim-2* nucleus became methylated (crossed-hatched rectangle) in the heterokaryon. (**B**) Southern analysis of methylation at *mtr*^{RIP8} in the *dim-2*; *mtr*^{RIP8}/*Δmtr* heterokaryon. Heterokaryons were formed by mixing N534 (*col-4 mtr*^{SR62}; *trp-2*) and N1931 (*mtr*^{RIP8}; nic-*3 wc-1 dim-2*^{1}) in liquid minimal medium in the indicated ratios. Genomic DNA was purified, digested with either *Dpn*II (D) or *Sau*3AI (S), fractionated, blotted and hybridized to an *mtr* probe. Lanes 1 and 2, DNA sample from N1930 (a control for methylation at *mtr*^{RIP8} in *dim-2*$^{+}$ background); lanes 3 and 4, DNA sample from N1931; lanes 5–12, DNA sample from heterokaryons; lane 13, DNA sample from N534. All the bands except for the two that also show up in *Dpn*I digests (e.g. lane 1) reflect methylation. The position of size standards (kb) is indicated on the left.

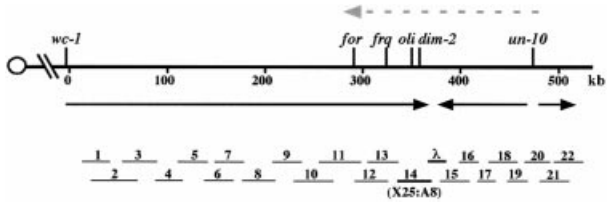

Fig. 2. Chromosome walk to the *dim-2* gene. The physical map of LG VIIR is shown with the centromere indicated by the open circle. The inverted order of the *for*, *frq*, *oli*, *dim-2* and *un-10* loci, relative to a previous map, is indicated by the dashed gray arrow above the map. Arrows below the physical map show the extent and orientation of our chromosomal walks from *wc-1* and *un-10*. Line segments below the map indicate positions of the key cosmid (1–22) and λ clones identified in the *dim-2* chromosome walk. The identities of all clones are listed in Materials and methods.

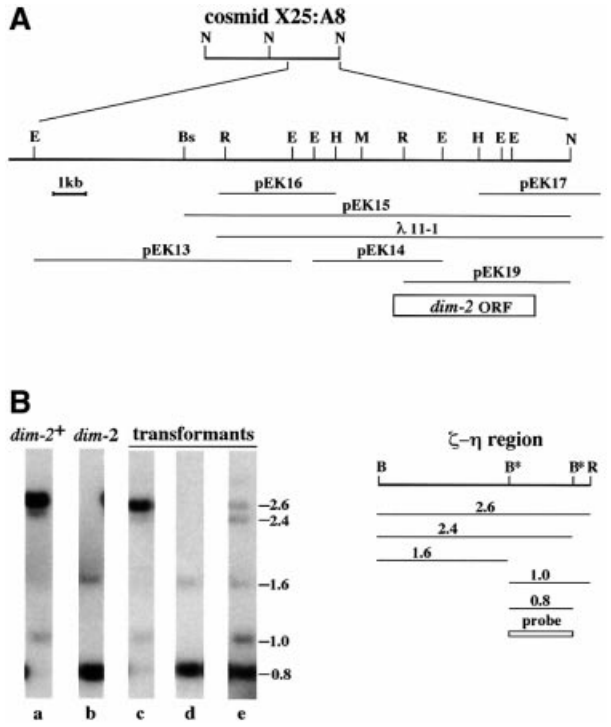

Fig. 3. Complementation of *dim-2* mutation. (**A**) Physical map of cosmid X25:A8 and related DNA segments. The positions of the *dim-2* ORF (open box) and of restriction sites for *Not*I (N), *Eag*I (E), *Bsp*120 I (Bs), *Eco*RI (R), *Hin*dIII (H) and *Mlu*I (M) are shown. (**B**) Southern analysis of methylation in the ζ-η region. The positions of *Eco*RI (R) and *Bam*HI (B) restriction sites in the region are shown, and the size (kb) and origin of fragments that can be detected with the indicated probe are represented below the map. Sites that are sometimes methylated, and thus not digested, are marked with asterisks. *Eco*RI/*Bam*HI digests of DNA from the following strains were probed to assess methylation at the ζ-η *Bam*HI sites: a, wild-type (N150); b, *dim-2*^{1} (N1257); c–e, N1257 transformed with pEK15, pEK14 and pEK19, respectively.

Southern hybridization. Substantial, but incomplete methylation was detected in all of the heterokaryons, indicating that the *dim-2* allele can be complemented and that the product of the *dim-2* gene is not nucleus limited (Figure 1B, lanes 5–12). This result suggested that *dim-2*$^{+}$ nuclei generated by transformation should be detectable even if most of the nuclei in the *dim-2* strain were untransformed.

Mapping and positional cloning of the dim-2 gene

To isolate the *dim-2* gene by positional cloning, we first mapped it genetically. Linkage tests with various tester strains revealed that *dim-2* is linked to *csp-2* on linkage group (LG) VII (Kouzminova, 2000). Results of a three-point cross with *wc-1* and *arg-10* mapped *dim-2* between these markers on the right arm of LG VII (~10 cM to the right of *wc-1* and ~6 cM to the left of *arg-10*); other crosses confirmed that *dim-2* is between *wc-1* and *un-10*. Based on published genetic maps and contigs established in previous walks in the region (Cabibbo *et al*., 1991; Ballario *et al*., 1996), we initiated what should have been

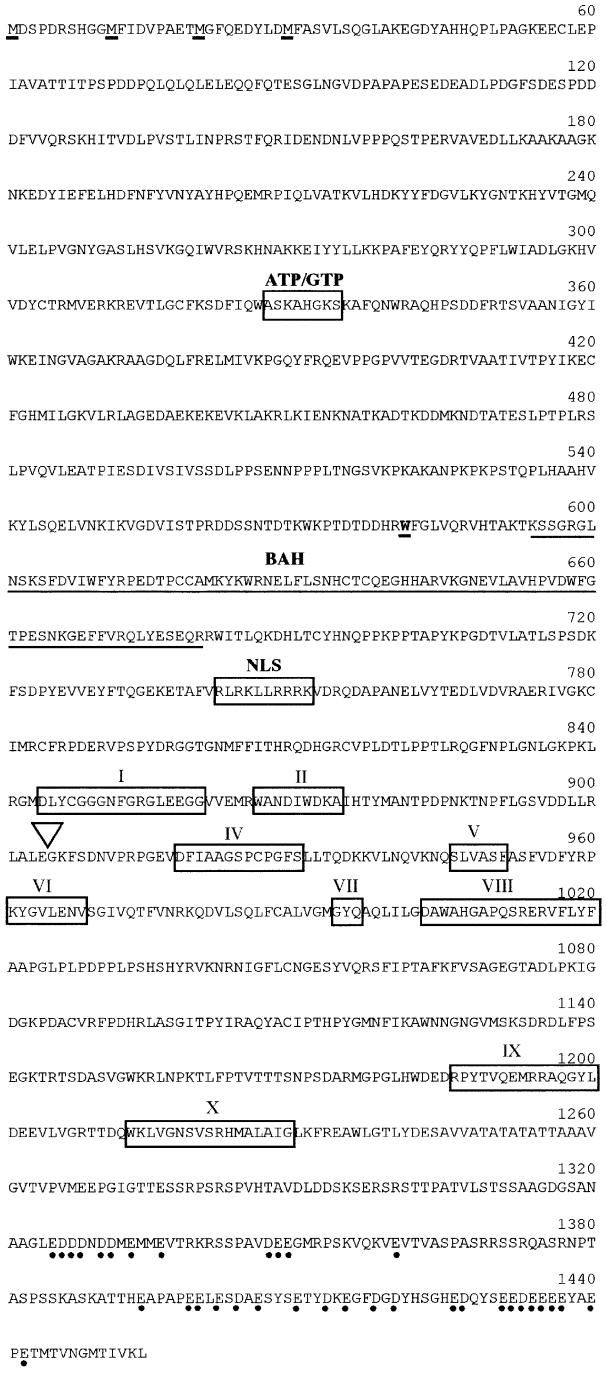

Fig. 4. The predicted amino acid sequence of the Dim-2 polypeptide. Four potential initiation methionines (M) are underlined. The boxed regions labeled with Roman numerals indicate motifs characteristic of DNA MTases (Kumar *et al*., 1994). A putative nuclear localization signal (NLS) and a nucleotide (ATP/GTP) binding motif are also boxed, and a bromo adjacent homology (BAH) domain (Callebaut *et al*., 1999) predicted by PFAM and SMART protein prediction web servers is underlined. The position of the only intron in the gene is indicated by the inverted triangle. The tryptophan (W) at amino acid position 581 that is mutated to a stop codon in the original *dim-2*[1] allele is shown in bold and underlined. The acidic amino acids in the C-terminus are indicated by dots.

converging chromosome walks from *wc-1* and *un-10*. We discovered, however, that the previously published genetic map has an inversion between *un-10* and *for* (Kouzminova and Selker, 1999). This led us to reverse the orientation of our chromosome walk from *un-10* (Figure 2). We covered the 550 kb interval between *wc-1* and *un-10* and confirmed that the previously published genetic map is incorrect—the correct order of the genes in the region being *wc-1*, *for*, *frq*, *oli*, *un-10*. Before attempting to locate *dim-2* by complementation, we assigned the gene to an individual cosmid within the contig by RFLP mapping as described in Materials and methods. Our results indicated that *dim-2* must be either at the very right end of cosmid X25:A8 or on lambda clone λ25:A8 (Figure 2, step 14 and λ).

Identification of the dim-2 gene by complementation

We transformed a *dim-2* strain (N1257) with cosmid X25:A8 and analyzed 20 transformants by Southern hybridization to test for methylation at two *Bam*HI sites that are normally methylated in the zeta-eta (ζ-η) region (Selker *et al*., 1987b) (Figure 3B). Two transformants showed partial restoration of methylation at both *Bam*H1 sites in the region, confirming that this cosmid contains the *dim-2* gene (data not shown). To map further *dim-2*, we divided X25:A8 into two 18–20 kb *Not*I fragments (Figure 3A) and introduced them separately into the *dim-2* strain. Two of 12 transformants generated with the larger *Not*I fragment showed unambiguous complementation of the methylation defect. The complementing fragment was further subcloned to yield plasmids pEK13, pEK14, pEK15 and pEK19 (Figure 3A). We also tested for complementation with lambda clones λ25:A8 and λ11-1. The latter gave complementation and was subcloned to generate plasmids pEK16 and pEK17. Full complementation was achieved in transformants with either pEK15 or λ11-1 (Figure 3B, compare lanes a and c), while partial complementation, revealed as weak 2.6 and 2.4 kb bands, was found with pEK19 (Figure 3B, compare a and e).

The dim-2 gene encodes a putative DNA (cytosine 5) methyltransferase

We sequenced the 5 kb insert in pEK19, and the adjacent 4 kb of DNA in pEK15, and found an open reading frame (ORF) with a region of strong similarity to the catalytic domains of known eukaryotic and prokaryotic MTases (Figure 4). The sequence of the *dim-2* gene suggested that it contains a single 64 nucleotide intron. The 5′- and 3′-splice junctions, as well as the internal sites of the predicted intron, fit the consensus for *N.crassa* introns (Bruchez *et al*., 1993a). Using a cDNA library made from conidial RNA (Nelson *et al*., 1997), we identified and sequenced cDNA clones for the gene, confirming that the ORF is transcribed and that the predicted intron is indeed absent from the polyadenylated mRNA (data not shown). Translation of the *dim-2* transcript probably initiates at the first ATG of the ORF. An in-frame stop codon is located 18 nucleotides before this codon, and the sequence surrounding it is consistent with the conserved context of the known initiator codons in *N.crassa* (Bruchez *et al*., 1993b). Three other candidate translation initiator codons are found near the beginning of the ORF (underlined in Figure 4).

The presumptive Dim-2 protein contains 1454 amino acids (160 kDa), including a C-terminal 600 amino acid segment that appears to correspond to the catalytic domain

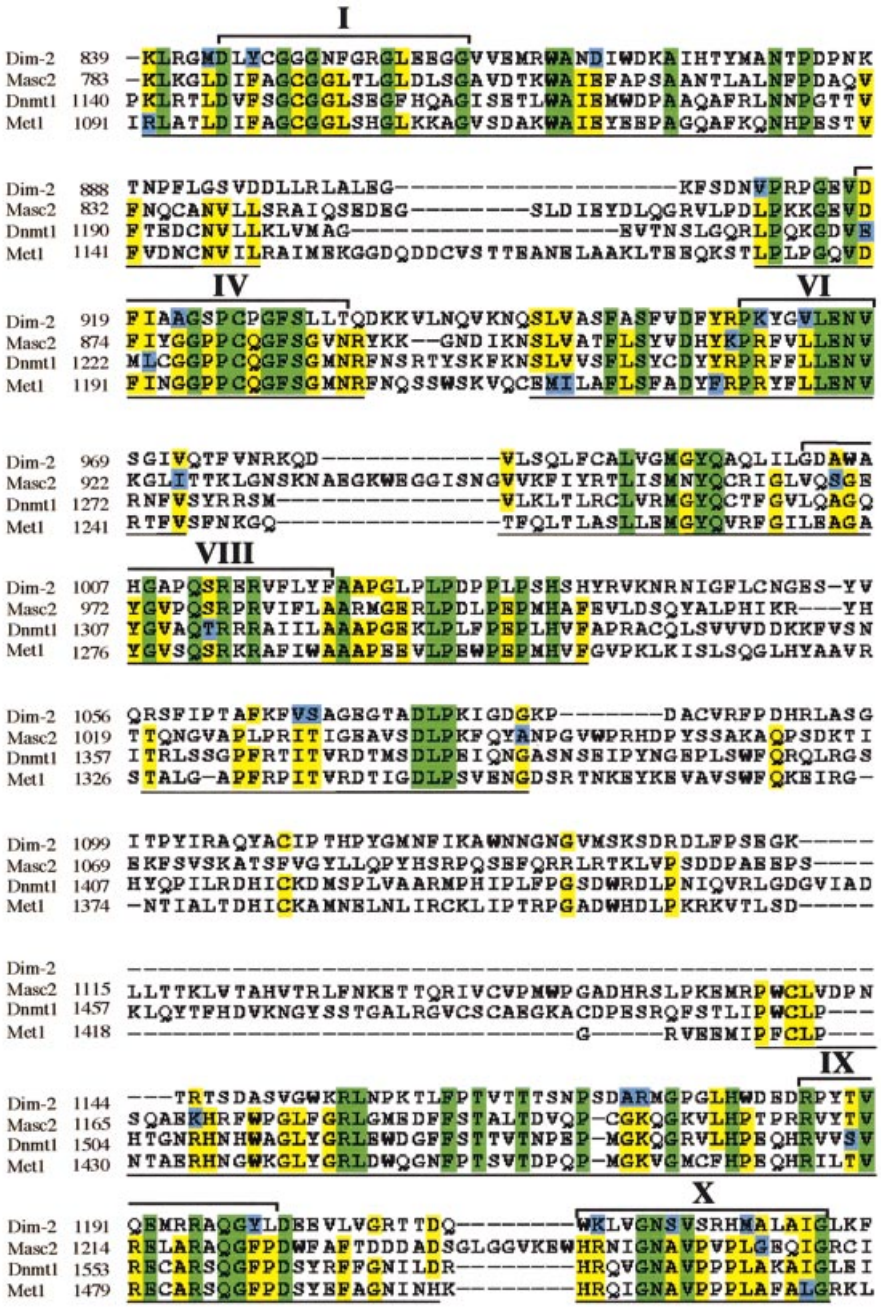

Fig. 5. Sequence alignment of the C-terminal domains of *Mus musculus* Dnmt1 (DDBJ/EMBL/GenBank accession No. P13864), *Ascobulus immersus* Masc2 (accession No. CAB09661), *Arabidopsis thaliana* MET1 (accession No. P34881) and *Neurospora crassa* Dim-2 (accession No. AF348971). Green boxes indicate amino acid identity in all four sequences, yellow boxes indicate amino acid identity in three out of four sequences, blue boxes indicate conservative changes, and dashes indicate gaps introduced by the CLUSTAL_W algorithm to maximize alignment (Thompson *et al*., 1994). The numbers on the left correspond to amino acid positions in the protein sequences. Six of the 10 conserved motifs defined previously based on prokaryotic MTases (Kumar *et al*., 1994) are indicated by brackets and Roman numerals above the aligned sequences. Pairwise BLAST 2 comparisons of the underlined regions demonstrated that Dim-2 is the most divergent MTase in the group. The expected values obtained from comparisons of Dim-2 to Masc2, Dnmt1 and MET1 are $3e^{-45}$, $1e^{-42}$ and $3e^{-44}$, respectively; expected values from comparisons of Masc2 to Dnmt1 and MET1 are $6e^{-84}$ and $5e^{-75}$, respectively; the expected value from the comparison of Dnmt1 to MET1 is $1e^{-110}$.

of known MTases (Figure 5). The six most conserved motifs (I, IV, VI, VIII, IX, X) of the 10 that characterize DNA MTases, as first defined with prokaryotic MTases (Posfai *et al*., 1989; Kumar *et al*., 1994), are prominent in the C-terminal domain (Figures 4 and 5). Sequence comparisons of the presumptive catalytic domains of known MTases, carried out using the BLAST 2 program (Tatusova and Madden, 1999), suggest that Dim-2 (amino acids 840–1454) is most similar to known and presumed MTases from plants, mammals and the fungus *A.immersus*. In the conserved regions of the C-terminal domain, Dim-2 shows 32–34% identity and 48–52% similarity, relative to the corresponding regions of *Ascobolus Masc2*, *Arabidopsis* MET1 and mouse

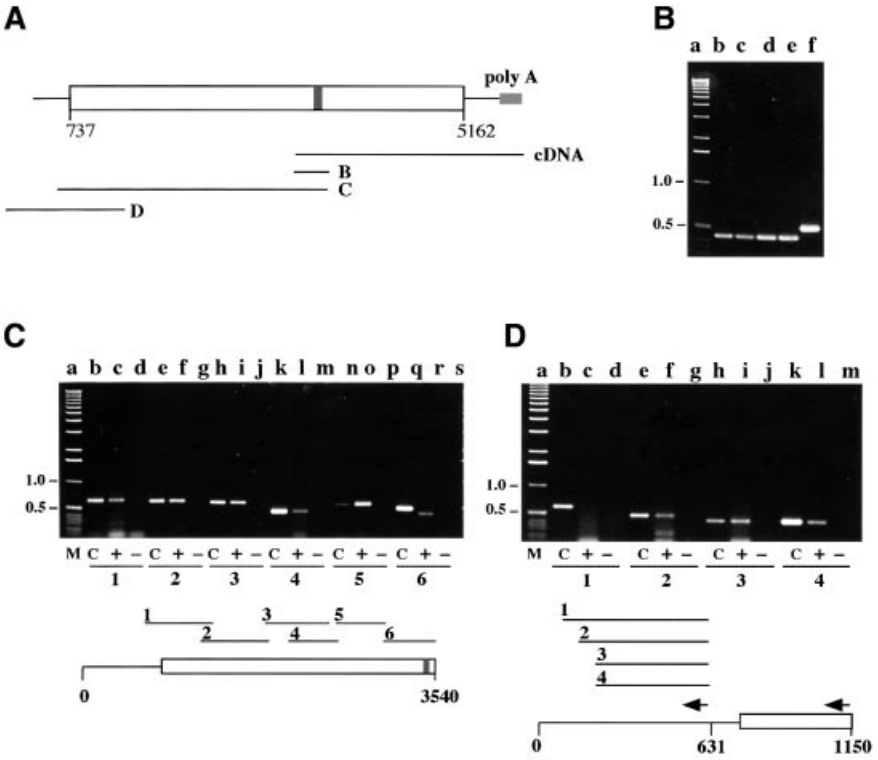

Fig. 6. Analysis of *dim-2* transcription by RT–PCR. PCR products were fractionated on 1.1% agarose gels. In each panel, the numbering of the sets of lanes indicates the gene segments tested, as illustrated below. In each set, 'C' (control) indicates that PCR was performed on genomic DNA, '+' indicates that RT was included in the RT reaction, while '–' indicates that RT was omitted. The primers are described in Materials and methods. (**A**) Diagram of the *dim-2* gene showing the ORF (starting at nucleotide 737 relative to the *Mlu*I site; open rectangle) and intron (nucleotides 3449–3512 relative to the *Mlu*I site; filled rectangle). Lines B, C and D represent regions tested by RT–PCR in the corresponding panels. The line marked 'cDNA' represents the longest identified *dim-2* cDNA. (**B**) Expression of *dim-2* in dormant conidia (lane b), and in conidia germinated for 3 (lane c), 7 (lane d) or 24 h (lane e). The RT reaction was performed with random hexamer primers using polyA-RNA from strain N150. The subsequent PCR was for the intron-containing region B, using primers #715 and #716. Lane f shows the PCR product from genomic DNA and lane a contains size markers. (**C**) Mapping the upstream portion of the *dim-2* transcript. RT reactions were performed with random hexamer primers. The following primer pairs were used for PCR products 1–6, respectively: #717/#692; #691/#728; #690/#684; #720/# 721; #693/#686; and #715/#716. (**D**) Mapping the 5′ end of the *dim-2* transcript. The RT reaction was performed with primer #713 (indicated by arrow left of position 631) and subsequent PCR reactions to generate products 1–3 were performed with primer pairs #713/#765, #713/#768 and #713/#712, respectively. Product 4 was obtained using primer #714 (indicated by arrow left of position 1150) in the RT reaction and primer pair #713/#712 for the subsequent PCR.

Dnmt1. Interestingly, these three MTases show 49–64% identity and 63–80% similarity among themselves (Figure 5). The N-terminal domains of known eukaryotic MTases are much less conserved than the C-terminal domains, but some MTases show similarities (Colot and Rossignol, 1999). The large N-terminal region of the predicted Dim-2 protein does not show significant similarity to any known protein in current databases, although it does show a few sequence features, including a bromo adjacent homology (BAH) domain (Callebaut *et al*., 1999) (amino acids 594–681), a putative nuclear localization signal (NLS), RLRKLLRRRK (amino acids 742–751) and a 'Walker A' ATP/GTP-binding motif (Walker *et al*., 1982) (amino acids 327–334; Figure 4).

Mapping the dim-2 transcript

We had a clue from our complementation tests that the N-terminal 186 amino acids of Dim-2 are not essential: pEK19 transformants with only 3.9 of the 4.4 kb Dim-2 ORF showed partial complementation of the *dim-2* mutation (sample transformant shown in Figure 3B). The partial complementation by pEK19 could reflect truncation of the Dim2 protein and/or absence of regulatory sequences from the transforming DNA. The pEK19 transformant shown in Figure 3 is one of two with the strongest complementation. No complementation was achieved when the same *dim-2* segment was targeted to the *his-3* locus (data not shown), consistent with the idea that transcription of the pEK19 sequences was driven by promoters that by chance were near the arbitrary genomic integration sites. As a step to determine whether the full *dim-2* ORF is expressed in wild-type cells, we investigated the length of the *dim-2* transcript. No signals were obtained for *dim-2* in northern hybridizations using as much as 20 μg of polyA-RNA prepared from a wild-type strain (N150) grown for either 7 or 24 h from conidia, whereas control probings for the *oli* and *am* genes produced strong hybridization signals (data not shown), suggesting that the *dim-2* transcript is rare. Further evidence that *dim-2* is not highly expressed came from screening cDNA libraries; only two *dim-2* clones, with 1.1 and 2.5 kb inserts, were found among 40 000 conidial cDNA clones (Nelson *et al*., 1997) screened, and only one clone (NMB5-9; 0.9 kb insert) was identified in *N.crassa* EST databases (see http://molbio.ahpcc.unm.edu/search/ngp.html). To determine whether the *dim-2* transcript is

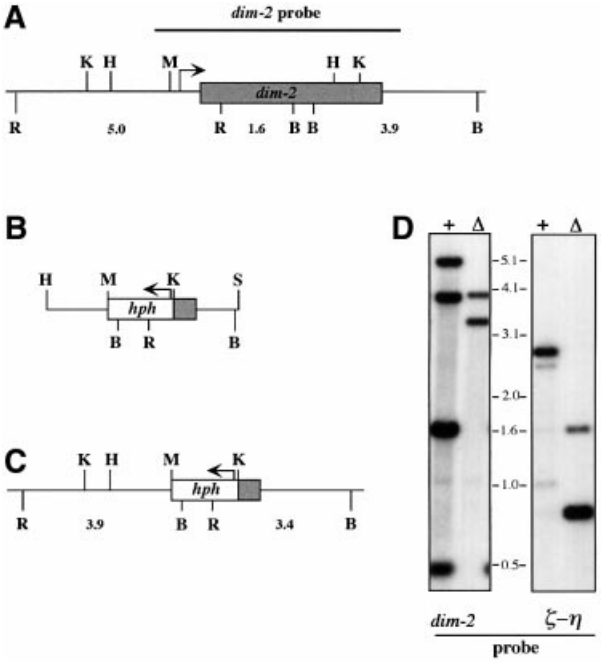

Fig. 7. Construction and characterization of the *Δdim-2²* allele. The gray and open bars represent the *dim-2* and *hph* ORFs, respectively, and the arrows indicate the direction of transcription. (**A**) Restriction map of the *dim-2⁺* allele showing sites for *Bam*HI (B), *Hin*dIII (H), *Kpn*I (K), *Mlu*I (M) and *Eco*RI (R), and various fragment lengths below (kb). (**B**) Diagram of the transforming *Sph*I (S)–*Hin*dIII fragment of pEK22 with most of *dim-2* replaced by the *hph* gene. (**C**) Structure of the resulting *Δdim-2²* allele. (**D**) Southern analysis of *dim-2⁺* and ζ-η regions in wild-type (N150; +) and *Δdim-2* (N1877; Δ) strains using *Eco*RI and *Bam*HI (see Figure 3).

long enough to encode the full predicted *dim-2* ORF, we performed RT–PCR using polyA-RNA from a wild-type strain (N150). The *dim-2* transcript was detected in non-germinated conidia (Figure 6B, lane b) as well as in growing vegetative cells (at least up to 24 h of growth; Figure 6B, lane e). Using random primers for the RT reaction, and a variety of PCR primers, we confirmed that the transcript includes the first ATG of the *dim-2* ORF (Figure 6C). Thus, the transcript is long enough to be translated into the longest predicted Dim-2 protein. To map the 5′ end of the message, we employed specific primers for RT reactions (Figure 6D). The RT–PCR analysis also confirmed the absence of additional introns in the ORF and leader of the *dim-2* transcript. The total length of the *dim-2* transcript is at least 5.3 kb and appears to include an ~700 nucleotide 5′ untranslated sequence and a 276 nucleotide 3′ untranslated sequence.

Characterization of the dim-2¹ allele and construction of a deletion allele

The unique, predicted large N-terminal domain of Dim-2 might confer a function distinct from the presumed MTase activity of the protein. This possibility prompted us to consider that the original mutation, *dim-2¹*, may not have eliminated all Dim-2 function(s) even though it appears to prevent all cytosine methylation. To address this possibility we isolated and sequenced the *dim-2¹* allele as described in Materials and methods. Analysis of nucleotides 431–5009 revealed a single, G→A mutation at nucleotide position 2449, ~40% into the ORF. The mutation changed the tryptophan codon at amino acid position 581 into a stop codon (Figure 4). Thus, the *dim-2¹* mutant should produce a truncated protein lacking the predicted catalytic domain and the putative NLS.

As a step to investigate further the function of the N-terminal segment of the Dim-2 protein, which is potentially expressed in the *dim-2¹* mutant, we constructed a *dim-2* null allele by gene replacement (Figure 7). To do so, we transformed *Neurospora* with a construct in which the N-terminal region, and most of the C-terminal domain of *dim-2*, was replaced with the bacterial *hph* gene driven by the *A.nidulans trpC* promoter (Cullen *et al*., 1987). We then screened 105 hygromycin resistant transformants by Southern hybridization and found several in which homologous recombination resulted in replacement of the native *dim-2* gene. The autoradiogram in the left panel of Figure 7D verifies the structure of one such strain (allele designated *Δdim-2²*), and the autoradiogram in the right panel demonstrates the absence of methylation in the ζ-η region. Examination of several other loci that are normally methylated [e.g. ψ63 (Margolin *et al*., 1998) and rDNA (Russell *et al*., 1985)], and also of total genomic DNA, by comparison of ethidium bromide-stained *Sau*3AI and *Dpn*II digests, confirmed the absence of methylation in this strain (data not shown). The *Δdim-2²* allele, like the *dim-2¹* allele, confers no obvious additional phenotypes in either the vegetative or sexual phase of the life cycle (see also 'RIP in the *dim-2* mutants' below). This suggests that the original *dim-2¹* mutant and the *Δdim-2²* mutant have equivalent defects.

Construction and analysis of Dim-2 variants

The striking similarity between the C-terminal 600 amino acid region of Dim-2 and known eukaryotic DNA MTases strongly suggests that Dim-2 is a DNA MTase, consistent with the phenotype of *dim-2* strains. It remained possible, however, that Dim-2 worked in a complex with an unknown DNA MTase that was directly responsible for the methylation defect of *dim-2* mutants. Attempts to detect the Dim-2 MTase using antibodies prepared against a segment of the protein produced in *E.coli*, or using epitope-tagged Dim-2, failed, perhaps because Dim-2 is present in very low levels in the cell, consistent with the low level of its mRNA. This hampered the testing of variant forms of the protein. The best we could do was to test all constructs as single copies, integrated by homologous recombination at a common chromosomal site under the regulation of a common promoter. We were able to verify that all constructs were expressed identically at the transcriptional level (data not shown). To test specifically that Dim-2 was a DNA MTase rather than a regulator of another MTase, we created and examined the effect of a single amino acid substitution in the putative catalytic site. The cysteine in the PC doublet of MTase motif IV serves as the catalytic nucleophile (Santi *et al*., 1983), as demonstrated, for example, by high-resolution structural data showing this cysteine linked to C6 of the cytosine subject to methylation (Klimasauskas *et al*., 1994). We therefore replaced cysteine 926 in the conserved PC with an alanine residue (construct C926A) and compared this strain (N1909) with a control strain (N1894) that was identical except that it lacked the amino acid

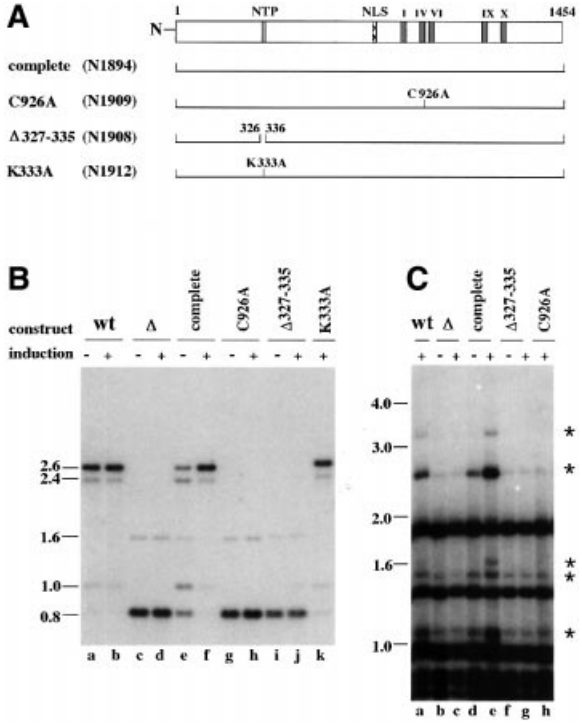

Fig. 8. DNA methylation in *Δdim-2* strains expressing various *dim-2* constructs. All mutant constructs were driven by the *N.crassa qa-2* promoter, which is inducible by quinic acid (Geever *et al*., 1989), and they were targeted to the *his-3* locus (Margolin *et al*., 1997) of strain N1877 (*Δdim-2*[2]) as single copies. All constructs contain 94 nucleotides upstream and 1 kb downstream of the *dim-2* ORF sequence. Strains were grown for 41 h in minimal medium with 1.5% sucrose in the presence (+) or absence (–) of 0.4% quinic acid as an inducer. Expression of all constructs was confirmed by RT–PCR (data not shown). (**A**) Structure of *dim-2* mutant constructs. The diagram at the top shows the position of an ATP/GTP binding motif (NTP), a putative nuclear localization signal (NLS) and the conserved MTase motifs of the Dim-2 protein (I, IV, VI, IX and X). The following Dim-2 constructs are shown: complete, full *dim-2* ORF; C926A, substitution of cysteine 926 with alanine in the conserved PC dipeptide of motif IV; Δ327–335, nine amino acid deletion removing the NTP binding motif, and K333A, substitution of lysine 333 with alanine. The numbering refers to amino acid positions relative to the predicted N-terminus. (**B**) Southern analysis of DNA methylation at the ζ-η locus in mutant constructs introduced into a *Δdim-2* host strain. DNA of wild-type control (N150; lanes a and b), the *Δdim-2* host strain (N1877; lanes c and d), a transformant with the complete gene (N1894; lanes e and f), a transformant with construct C926A (N1909; lanes g and h), a transformant with NTP binding motif deletion (N1908; lanes i and j) and a transformant with construct K333A (N1912; lane k) were digested with *Eco*RI and *Bam*HI and probed with the 0.8 kb *Bam*HI fragment from the ζ-η region (see Figure 3). (**C**) Southern analysis of rDNA using *Sau*3AI and a 9 kb *Kpn*I fragment carrying the entire rDNA unit as the probe. Lanes: a, wild-type strain (N150); b and c, *Δdim-2* host strain (N1877); d and e, transformant (N1894) with the complete construct; f and g, transformant (N1908) with a deletion of the NTP binding motif; and h, transformant (N1909) with C926A construct. The membrane was initially probed with a fragment of the *am* gene (Rountree and Selker, 1997) to verify complete digestion of all DNA samples with *Sau*3AI. Asterisks indicate bands that become more prominent due to methylation in strain N1894 under inducing conditions.

substitution (Figure 8A). No methylation was observed in the mutant strain, while full methylation was restored in the control strain (Figure 8B, compare lanes e and g with lane a, and f and h with lane b; Figure 8C, compare lanes e and h with lane a). A similar construct, bearing a different single amino acid substitution, also complemented the *dim-2* mutation, unlike the construct with the change in the presumed catalytic site (see below). This demonstration that substitution of a single amino acid in the putative catalytic site prevents methylation renders the idea that Dim-2 is a regulator of another MTase extremely unlikely.

To explore the possible importance of the ATP/GTP binding motif found in Dim-2, we first constructed a mutant (N1908, Δ327–335; Figure 8A) with a nine amino acid deletion including the motif (ASKAHGKS). Unlike control strains grown under identical conditions, the Δ327–335 construct did not rescue the methylation deficiency of the *dim-2* mutant (Figure 8B, compare lanes j and f; Figure 8C, compare lanes g and e), suggesting that the nine amino acid segment is essential for the activity, stability or conformation of Dim-2. To test more specifically the possible role of the nucleotide binding motif, we replaced the lysine in the GKS sequence with an alanine, generating construct K333A. Mutation analyses of NTP-binding domains in various proteins has revealed that this lysine is important for the coordination of the nucleotide (Hung *et al*., 1998; Iaccarino *et al*., 1998). The K333A construct restored methylation (Figure 8B, lane k), suggesting that NTP binding is not necessary for Dim-2 activity.

Table I. Testing effect of the *dim2*[1] allele on RIP

Cross	Relevant genotype[a]	RIP frequency (%)[b]	
		11 days[c]	17 days
N1259 × N1257	*dim-2*, *mtr*[+]/*mtr*[+] × *dim2*	18.9 ± 0.4 (135)	86.9 ± 5.4 (256)
N1255 × N1150	*mtr*[+]/*mtr*[+] × (+)	22.6 (47)	76.9 (52)
N1258 × N1150	*dim-2*, *mtr*[+]/*mtr*[+] × (+)	17.2 ± 5.7 (157)	80.8 ± 5.8 (208)
N1255 × N1257	*mtr*[+]/*mtr*[+] × *dim2*	34.6 (52)	80.8 (51)

[a]*dim-2* designates the *dim-2*[1] allele and *mtr*[+]/*mtr*[+] designates a duplication of *mtr* involving an ectopic copy of *mtr* plus the native allele. When not listed, the alleles of *dim-2* and *mtr* are wild type (+). The female strains carried the duplication.
[b]RIP frequency was calculated as a ratio of Mtr[–] progeny to a half of the total progeny. For crosses that were repeated several times, the average and standard error are shown. The number of progeny analyzed in the crosses is indicated in parentheses.
[c]Ascospores were harvested at the indicated time after fertilization.

Dim-2 is not involved in the mechanism of RIP

As discussed in the Introduction, the relationship between RIP and DNA methylation is not well understood. It is commonly assumed that the mutations by RIP result from methylation of cytosines followed by deamination, and it has been proposed that the deamination itself might be catalyzed by a MTase or a MTase-like enzyme (Selker, 1990). The Dim-2 MTase was an obvious candidate for one or both hypothetical activities. We therefore examined the possible involvement of Dim-2 in RIP by looking at inactivation of the *mtr* gene in crosses of *dim-2*[+] and *dim-2*[–] strains (N1255, N1258 and N1259) bearing an *mtr* duplication. The duplication was created by crossing an *mtr*[+] strain with a strain bearing a deletion of the native *mtr* gene but having an ectopic copy of *mtr*. The four possible

Table II. Testing effect of the *Δdim 2*[2] allele on RIP

Cross	Relevant genotype[a]	RIP (%)[b]	*hph* (%)[c]
N1879 × N1851	*Δdim-2*, *am/am* × *Δdim-2*	52	
N1879 × N150	*Δdim-2*, *am/am* × (+)	48	42
N1880 × N1851	*am/am* × *Δdim-2*	56	58
N1880 × N150[d]	*am/am* × (+)	72	
N1851 × N1879	*Δdim-2* × *Δdim-2*, *am/am*	56	
N150 × N1879	(+) × *Δdim-2*, *am/am*	24	38
N1851 × N1880	*Δdim-2* × *am/am*	40	44
N150 × N1880[d]	(+) × *am/am*	64	

[a]*Δdim-2* designates the *Δdim-2*[2] allele; *am/am* designates an *am* gene duplication resulting from an ectopic copy of *am* plus the native allele. When not listed, the alleles of *dim-2* and *am* are wild type (+). The female strains are listed first.
[b]RIP was calculated in all crosses for 50 random progeny as the ratio of Am$^-$ progeny to one half of the number of total progeny.
[c]Progeny were tested for independent segregation of the *hph* marker (expectation: 50%), which was inserted at *dim-2* in building the *dim-2* deletion allele.
[d]Progeny from crosses with the strain N1880 were tested by Southern analysis for RFLP with *Bam*H1 and *Eco*RI to verify the occurrence of RIP.

crosses between strains carrying the *dim-2*$^+$ or *dim-2*^{1} alleles were performed (Table I). Because the frequency of RIP is known to be low in ascospores produced early (Singer *et al*., 1995a), we collected and analyzed spores produced at both 11 days ('early') and 17 days ('late') after fertilization. Duplications of *mtr* were inactivated by RIP at a frequency of 18–34% among early ascospores, compared with 77–87% among late ascospores (Table I). No effect of the *dim-2*^{1} allele on the frequency of RIP was detected at either time point, even in crosses homozygous for *dim-2*^{1}. Since the *dim-2*^{1} allele can not encode a functional MTase, it seems unlikely that the Dim-2 MTase participates in either of the postulated steps of RIP.

To address the possibility that Dim-2 is involved in RIP through its N-terminal domain, which may be produced by the *dim-2*^{1} allele, we constructed an unlinked duplication of *am* in strains that had either the wild-type or the Δ*dim-2*^{2} allele (strains N1879 and N1880). We crossed *am* non-duplication strains with strains carrying the *am* duplication in various combinations (Table II). RIP of the *am* duplication was assayed by loss of *am* function, and progeny from each of two crosses were tested for RFLPs resulting from RIP. In control crosses, in which both parents had the *dim-2*$^+$ allele, the RIP frequency was 64 and 72% in ascospores produced 20 days post-fertilization. The frequency of RIP was 24–56% in crosses heterozygous for the Δ*dim-2* allele and 52–56% in crosses homozygous for this null allele (Table II). The observed variation in RIP frequency is expected from variations in the strain backgrounds (our unpublished results). The lack of a noticeable effect of the *dim-2* deletion on RIP is consistent with the results from the *mtr* duplication and *dim-2*^{1} allele. We conclude that Dim-2 is not involved in the mechanism of RIP.

Discussion

Although the function of DNA methylation is not fully understood in any eukaryote, it is clearly responsible for gene silencing in a variety of organisms, including *Neurospora* (Cambareri *et al*., 1996; Irelan and Selker, 1997; Rountree and Selker, 1997; Zhou *et al*., 2001). The *Neurospora dim-2* mutant is the only eukaryotic mutant presently known that appears to be completely devoid of DNA methylation (Foss *et al*., 1993). We cloned the *dim-2* gene to identify its product, which was clearly a key component of the DNA methylation machinery. To do so, we first mapped the locus to the right arm of LG VII between *wc-1* and *un-10* and built a 550 kb contig spanning the *wc-1* to *un-10* interval. During the course of this work we uncovered an inversion of the published genetic map between *un-10* and *for*. After localizing the *dim-2* gene to a small region by RFLP mapping, we identified it unambiguously by complementation. Sequencing revealed a 1454 amino acid ORF ending in a 600 amino acid domain showing obvious homology to known prokaryotic and eukaryotic DNA MTases. Results from RT–PCR analyses indicated that the *dim-2* transcript is at least 5.3 kb long, consistent with the long N-terminal region of the ORF. Other known eukaryotic DNA MTases also have substantial N-terminal domains that are not well conserved, unlike their C-terminal domains, e.g. mammalian DNMT1 MTases have an ~1000 amino acid N-terminal domain (Colot and Rossignol, 1999). Work in the last few years has identified several proteins that interact with the N-terminal domain of mouse and/or human (Yen *et al*., 1992) DNMT1, including the replication processivity factor PCNA (proliferating cell nuclear antigen; Chuang *et al*., 1997), histone deacetylases HDAC1 (Fuks *et al*., 2000; Robertson *et al*., 2000) and HDAC2 (Rountree *et al*., 2000), the tumor suppressor protein Retinoblastoma (Rb; Robertson *et al*., 2000), the transcriptional activator E2F1 (Robertson *et al*., 2000), the transcriptional co-repressor DMAP1 (Rountree *et al*., 2000) and the methyl-binding domain protein MBD3 (Tatematsu *et al*., 2000). Curiously, the N-terminal part of the *dim-2* ORF shows no significant similarity to any gene in current public databases. Analysis of mutant *dim-2* alleles constructed *in vitro*, and targeted precisely to the *his-3* locus, suggested that MTase function is abolished by an alanine to cysteine substitution in the presumptive catalytic domain but not by a single amino acid substitution at the conserved lysine in a putative NTP-binding motif. A deletion that would remove 186 amino acids from the N-terminus of the predicted protein did not abolish function. In contrast, deletions of the entire N- or C-terminal domains did not give detectable *dim-2* function (our unpublished observations). Although we demonstrated equivalent expression of all constructs at the transcriptional level, we did not succeed in producing antibodies that could recognize the Dim-2 MTase, or derivatives thereof. Thus, it is possible that the segments of Dim-2 that failed to function *in vivo* were unstable or not properly localized. Nevertheless, it is interesting that our results are consistent with reports that the C-terminal domain of the Dnmt1 MTase, when expressed alone in COS-7 cells, lacks catalytic activity (Zimmermann *et al*., 1997; Margot *et al*., 2000). The bulk of the N-terminal domain of Dnmt1, except for the first 300 amino acids, seems to be required for methyltransferase activity, e.g. as assayed *in vitro* with a poly(dI–dC) template.

Notable sequence features of the Dim-2 MTase in addition to the predicted catalytic site of MTase motif IV and the 'Walker A' (NTP-binding) motif, include MTase motifs I, VI, IX and X, a putative nuclear localization signal (NLS) and a bromo-adjacent homology (BAH) region (Callebaut *et al*., 1999) in the N-terminal region. Except for the putative NTP-binding site, these motifs have been found in several other known, and presumed, DNA MTases (Colot and Rossignol, 1999). It is worth noting that the *dim-2* BAH domain is a worse match to the consensus sequence (expected value: $9e^{-5}$) than those of mouse Dnmt-1 (expected value: $4.8e^{-37}$), *Arabidopsis* Met-1 (expected value: $1.5e^{-55}$) and *Ascobolus* Masc-2 (expected value: $1e^{-44}$). Dim-2 lacks other obvious motifs found in some eukaryotic MTases such as an PCNA-binding motif and dipeptide (e.g. GK) repeats. Unlike some MTases, Dim-2 has a 220 amino acid segment downstream of the conserved MTase motifs (Kumar *et al*., 1994; Colot and Rossignol, 1999). This segment shows no obvious homology to other known proteins, but, like the C-terminus of *Masc2* of Ascobolus (Chernov *et al*., 1997), one of the closest relatives of Dim-2 currently known, this segment is rich in acidic amino acids (Figure 4).

Of the approximately two dozen eukaryotic DNA MTases and putative DNA MTases described to date, Dim-2 appears to be the most divergent. The length of the N-terminal region and the sequence of the C-terminal region make Dim-2 most similar to the Dnmt1 subfamily of DNA MTases, which are regarded as maintenance MTases (Colot and Rossignol, 1999). The Dim-2 N-terminus lacks significant sequence homology with N-termini of other described Dnmt1-like MTases, however. Furthermore, as mentioned above, Dim-2 lacks the dipeptide repeats that link the N- and C-terminal domains of all previously described Dnmt1-like MTases. Thus, the lack of homology in its N-terminal domain and its lack of repeats distinguishes Dim-2 from the MTases of the Dnmt1 subfamily. Moreover, pairwise comparisons of the conserved regions of Dim-2 and representatives of the most closely related DNA MTases (*Arabidopsis* MET1, mouse Dnmt1 and *Ascobolus Masc2*) using BLAST 2, revealed that Dim-2 is substantially less similar to these MTases than they are to each other (Figure 5). The expected values obtained from comparisons of Dim-2 to Masc2, Dnmt1 and MET1 are $3e^{-45}$, e^{-42} and $3e^{-44}$, respectively, whereas comparisons of Masc2 and Dnmt1, Masc2 and MET1, and Dnmt1 and MET1 gave expected values of $6e^{-84}$, $5e^{-75}$ and $1e^{-110}$, respectively. The observation that Dim-2 does not appear more similar to the fungal MTase (Masc2) than known plant MTases (especially that from carrot; DDBJ/EMBL/GenBank accession No. AF007807), suggests that *Masc2* is not a true ortholog of *dim-2*. Consistent with this hypothesis is the observation that disruption of Masc2 does not affect methylation in *Ascobolus* (Malagnac *et al*., 1999).

Our finding that Dim-2 is responsible for all apparent DNA methylation in *Neurospora* raises the possibility that this single MTase is capable of both *de novo* and maintenance methylation. This contrasts with the situation in the mammals and plants that have been examined, which each sport several MTases, consistent with the idea that DNA methylation patterns in higher eukaryotes are established and propagated by separate enzymes. Although it is not certain that any eukaryotic DNA MTase is exclusively devoted to *de novo* or maintenance methylation, biochemical and genetic data have provided evidence that some MTases, such as Dnmt1 in mammals, are devoted primarily, or exclusively, to maintenance methylation, while others, such as Dnmt3a and Dnmt3b in mammals, are devoted to *de novo* methylation (Bestor, 1992; Okano *et al*., 1998; Hsieh, 1999; Lyko *et al*., 1999; Okano *et al*., 1999; Howell *et al*., 2001). It is generally assumed that maintenance methylation depends on the symmetry of methylated sites (e.g. CpGs in mammals and CpGs and CpXpGs in plants), and that methylation at non-symmetrical sites, such as is prevalent in *Neurospora*, is due to *de novo* methylation (Selker and Stevens, 1985; Selker *et al*., 1987b). This view is supported by a variety of observations, including the recent observation of non-CpG methylation by the candidate *de novo* MTase Dnmt3a (Ramsahoye *et al*., 2000). Nevertheless, there are clues that this view is overly simplistic. For example, the strict maintenance model does not easily account for the heterogeneity in methylation patterns commonly observed in plant, animal and fungal systems.

The fact that mutations in *dim-2* promptly and completely eliminate DNA methylation, and that methylation is promptly restored after introduction of *dim-2*$^+$ DNA into *dim-2* strains, argues that Dim-2 is a *de novo* MTase. Nevertheless, Dim-2 may also be capable of maintenance methylation. Evidence of maintenance methylation in *Neurospora* has come from two studies. In a survey of *am* alleles generated by RIP, some exceptional, lightly mutated, methylated alleles were found that were not subject to *de novo* methylation after demethylation by either of two methods (Singer *et al*., 1995b). A similar observation was made with the bacterial antibiotic resistance gene *hph* located between copies of *am* that had been subjected to RIP (Irelan and Selker, 1997). Re-establishment of methylation (and silencing) was incomplete after treatments causing demethylation, unlike the situation with most methylated sequences in *Neurospora*, such as sequences heavily mutated by RIP. The implication was that some of the original methylation of *hph* was due to maintenance methylation. In both cases, the methylation was established in the sexual cycle and was stably maintained in vegetative cells, but could not be re-established in vegetative cells after demethylation. The methylation was not limited to symmetrical sites, implying that methylation can promote subsequent methylation in a different way than that predicted by the original maintenance methylation model (Holliday and Pugh, 1975; Riggs, 1975). The fact that residual methylation has not been observed in a *dim-2* strain isolated from a Dim$^+$ *dim-2*/*dim-2*$^+$ heterokaryon (M.Freitag and E.U.Selker, unpublished observation) suggests that the Dim-2 MTase may be responsible for both maintenance and *de novo* methylation in a variety of sequence contexts. Biochemical studies on Dim-2 should help to define the substrate specificity of this enzyme and detailed genetic studies should help to identify functions of its long N-terminal domain and acidic C-terminal tail.

The relationship between cytosine methylation and RIP is intriguing but not fully understood. What is certain is that many sequences mutated by RIP serve as excellent substrates for *de novo* methylation *in vivo*. Evidence of

Table III. *Neurospora* strains

Name	Genotype	Source[a]
N1	*a*	FGSC 988
N51	*A*	FGSC 2225
N150	*A*	FGSC 2489
N268	*am*^{132} *am*$^{ecT–510\ 5.6}$ *inl*; *a*	Selker *et al.* (1988)
N534	*col-4 mtr*^{SR62}; *trp-2*; *a*	D.Stadler
N581	*col-4 mtr*^{SR33}; *(mtr+/hph+)*^{ecpAH33}; *trp-2*; *a*	lab collection
N623	*his-3*; *A*	FGSC 6103
N1150	*lys-1*; *A*	lab collection
N1255	*(mtr+/hph+)*^{ecpAH33}; *a*	this study
N1257	*dim-2*^{1}; *A*	lab collection
N1258	*(mtr+/hph+)*^{ecpAH33}; *dim-2*^{1}; *a*	this study
N1259	*(mtr+/hph+)*^{ecpAH33}; *dim-2*^{1}; *a*	this study
N1847	*wc-1 frq*^{10} *dim-2*^{1} *arg10*; *a*	this study
N1850	*Δdim-2*^{2}; *a*	this study
N1851	*Δdim-2*^{2}; *A*	this study
N1877	*Δdim-2*^{2}; *his-3 a*	this study
N1879	*am*$^{ecT–510\ 5.6}$; *Δdim-2*^{2}; *a*	this study
N1880	*Am*$^{ecT–510\ 5.6}$; *a*	this study
N1892	*Δdim-2*^{2}*; his-3*$^{+}$::*dim-*$^{2pEK20–4}$ *a*	this study
N1894	*Δdim-2*^{2}; *his-3*$^{+}$::*dim-*$^{2pEK24–1}$ *a*	this study
N1908	*Δdim-2*^{2}; *his-3*$^{+}$::*dim-*$^{2pEK30–12}$ *a*	this study
N1909	*Δdim-2*^{2}; *his-3*$^{+}$::*dim-*$^{2pEK32–2}$ *a*	this study
N1912	*Δdim-2*^{2}; *his-3*$^{+}$::*dim-*^{2pEK34} *a*	this study
N1930	*mtr*^{RIP8}; *lys-1A*	this study
N1931	*mtr*^{RIP8}; *nic-3 wc-1 dim-2*^{1}; *a*	this study

[a]Strains with FGSC designations are from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, KS 66103).

methylation induced in the sexual phase of the *Neurospora* life cycle is consistent with the possibility of an additional connection between RIP and methylation, namely that the mechanism of RIP involves methylation of cytosines. Unfortunately, because of technical difficulties involved in analyzing the minuscule ascogenous tissue in which RIP takes place, it has not yet been possible to determine unequivocally whether or not methylation occurs coincidently with RIP. Curiously, preliminary analysis of DNA methylation in the ascogenous tissues indicates that at least some sites in the sequences that are methylated in vegetative tissues lose methylation during the sexual cycle (data not shown). Moreover, we presented genetic evidence indicating that the Dim-2 MTase does not participate in the RIP mechanism. As pointed out previously, based on chemical principles, it seems possible that RIP results from deamination of cytosines catalyzed by an MTase-like protein (Selker, 1990). Perhaps a sexual phase-specific MTase, or MTase-like protein, is responsible for RIP. Support for this possibility comes from the discovery of *Masc1*, a putative MTase in the fungus *A.immersus* that appears to play a role in the RIP-like process called MIP, but which does not appear to play a role in DNA methylation in vegetative cells (Malagnac *et al*., 1997). A *Neurospora* homolog of *Masc1* has recently appeared in an *N.crassa* database (DDBJ/EMBL/GenBank accession No. AL442164.1) and experiments are under way to determine whether or not this gene plays a role in RIP. The *Neurospora* genome sequence is nearly complete, and no additional DNA MTase-related protein is found in the database (http://waldo.wi.mit.edu/annotation/fungi/neurospora), consistent with our conclusion that *dim-2* is responsible for all known methylation in this organism.

Materials and methods

Strains, media, growth conditions and transformation procedures

Neurospora strains used in this study are described in Table III and were handled according to standard procedures (Davis, 2000). In general, liquid cultures of *Neurospora* were grown at 32°C for 2 days with vigorous shaking in Vogel's medium (Davis, 2000) with 1.5% sucrose and required supplements. For quinic acid induction of $P_{qa\text{-}2}$*dim-2* constructs, 30 ml liquid cultures were grown from an inoculum of 1×10^6 conidia/ml for 41 h at 32°C with shaking (130 rpm) in Vogel's medium with 1.5% sucrose and 0.4% quinic acid pH 6. Hygromycin B (Calbiochem) was used at 200 μg/ml to select for the *hph* gene (Staben *et al*., 1989). Scoring for the following *N.crassa* genes was as described previously: *wc-1*, *un-10* and *arg-10* (Perkins *et al*., 1982); *am* (Kinsey *et al*., 1980); and *mtr* (Irelan *et al*., 1994).

Escherichia coli strains were normally grown in liquid or solidified Luria broth supplemented with ampicillin (200 μg/ml) as necessary. Plasmids were propagated in *E.coli* strain DH5αF′. Phage lambda was propagated in *E.coli* strain A585 (C600 *recD1009* SuII SuIII; kindly provided by Frank Stahl, University of Oregon), grown in tryptone broth according to standard procedures (Ausubel *et al*., 1998).

Transformation of *Neurospora* strain N1257 was performed by electroporation (Margolin *et al*., 1997) using 1 μg of cosmid DNA or, for cotransformation experiments, a mixture of 0.25 μg of pCSN43 (Staben *et al*., 1989) and 1 μg of lambda DNA or 0.25 μg of pEK19. Transformants were isolated and propagated on minimal medium supplemented with hygromycin. To target DNA to the *his-3* locus of strains N1877, plasmids were linearized with *Afl*III. Successful gene replacements were verified and analyzed by Southern analysis.

Neurospora crassa recombinant DNA libraries

Two *N.crassa* genomic DNA libraries were used primarily in the chromosome walk: the Orbach/Sachs cosmid library (Orbach, 1994) and a λJ1 library provided by the Fungal Genetic Stock Center (FGSC; University of Kansas Medical Center, Kansas City). These, as well as conidial (C1) and mycelial (M1) cDNA libraries (Nelson *et al*., 1997) were obtained from the FGSC. Cosmid libraries were replica-plated on LB plates covered with nylon membranes (Hybond-N; Amersham Pharmacia Biotech). Membranes with *E.coli* colonies or phage plaques were processed as recommended by the manufacturer. The cosmids and lambda clones identified in the *dim-2* chromosome walk and described in Figure 2 are: 1, G7:G3; 2, G21:F8, G14:C1, 56:E7[a]; 3, X11:G8, X14:B3; 4, X3:B5, G11:G3; 5, G23:F12, G18:H18; 6, G12:D7, G3:C9, G5:C10; 7, G4:A3, G9:E9, X4:C5, X14:A5; 8, X9:G1, X3:A4, X6:G10, X12:C2; 9, X13:G3, X17:G9, X17:F12, G14:F11, G20:D9, G23:F9; 10, X5:G3, X15:D7, X19:H11, G6:G3; 11, G19:D1, G10:H7, X16:C9; 12, X15:E7; 13, G12:B8, G1:C1, G15:B2, G20:A9, G22:F2, G22:C11, X21:F1; 14, X25:A8; 15, cos2-10A[b]; 16, G2:F8, X18:D6; 17, G1:H8, X20:E3; 18, G6:H11, G19:C12, X20:E8; 19, G17:D7, G24:F3, G2:F3; 20, cos10:12E[b], G17:C7, X20:C9; 21, G17:C9, X8:H8, 55:E3[a]; 22, X10:C4, X24:C10, G7:F11, G16:E5, 26:A8[a], λ– λ25:A8, λ3–1, λ7–1, λ11–1. All cosmids were from the Orbach/Sachs library (Orbach, 1994), except those marked with a or b superscripts, which are from the CBM1 (Cabibbo *et al*., 1991) and Vollmer/Yanofsky (Vollmer and Yanofsky, 1986) libraries, respectively.

RFLP mapping

At each step in the chromosome walks, the cosmid chosen to be the next probe was RFLP mapped using 38 meiotic progeny from ordered asci from a cross between Oak Ridge (OR) and Mauriceville (M) strains (Metzenberg *et al*., 1985), to verify that it was specific for LG VIIR. DNA samples were digested with *Eco*RI, fractionated by gel electrophoresis and probed with radiolabeled total cosmid DNA. To assign a given cosmid to a particular chromosomal region, its pattern of inheritance was compared with that of previously mapped sequences on the seven chromosomes of *Neurospora* (Nelson and Perkins, 2000).

The position of *dim-2* in the cosmid contig was determined by employing 45 recombinant strains in the *frq* to *arg-10* interval from a cross between an OR lineage strain (N1847) and an M lineage strain (N51; Table III). DNA prepared from these strains was digested with *Eco*RI, *Bgl*II, *Hin*dIII or *Dpn*II and analyzed by Southern hybridization

with probes from cosmid or lambda clones in the region. Two recombinants proved to be the most informative: #12, which is *wc-1 frq*^{10} *dim-2*$^{+}$*arg-10*$^{+}$ and showed M-type RFLPs to the right of *oli*, indicating that the recombination occurred close to *oli*, and that the *dim-2* gene is to the right of *oli*; #16, which is *wc-1 frq*^{10} *dim-2*^{1} *arg-10*$^{+}$ and showed M-type RFLPs to the right of step 15 of the walk, placing *dim-2* to the left of step 15 (Figure 2).

Nucleic acid isolation, DNA hybridization and RT–PCR analysis

Plasmids and cosmids were purified by standard procedures such as the alkaline lysis procedure (Birnboim and Doly, 1979). Lambda DNA was isolated as described elsewhere (Kouzminova, 2000). *Neurospora* genomic DNA was isolated as described previously (Foss *et al*., 1993), as was total RNA (Luo *et al*., 1995). Poly(A)-RNA was prepared using the PolyATract mRNA Isolation System III (Promega).

Southern hybridization analyses were performed as described previously (Irelan and Selker, 1997) using DNA probes prepared by priming with random hexamers (Feinberg and Vogelstein, 1984).

For RT reactions, 1 µg of poly(A)-RNA or total RNA was first treated with DNAse I according to the protocol of the manufacturer (Gibco-BRL). First-strand cDNA synthesis was performed at 37°C for 60 min with MMLV RT according to the protocol of the supplier (Promega). PCR was carried out in 100 µl of 1× Promega *Taq* polymerase buffer supplemented with 1.5 mM $MgCl_2$, 200 µM dATP, dCTP, dGTP and TTP, 0.4 µM of each primer, 2.5 U of *Taq* polymerase (Promega) and either 10 ng of plasmid DNA or 100 ng of genomic DNA. PCR were carried out using a Hybaid Omnigene thermocycler as follows: 94°C for 4 min, followed by 30 cycles of [94°C for 4 s, 50–54°C for 10 s, 72°C for 30 s per 1 kb], followed by 72°C for 5 min. *Pfu* polymerase was used according to the protocol of the manufacturer (NEB). PCR reactions described in Figure 6 were carried out with the following primer pairs: #717/#692, 5′-GCCATGGATTCGCCAGATCGC-3′/5′-CTTGGTCGC-TACAAGCTG-3′; #691/#728, 5′-CATTGATCTCCTTCCAGA-3′/5′-TACCGCCTCCGCAGTCAAC-3′; #690/#684, 5′-GTTTGAACTGTC-ATCTCG-3′/5′-TCTGGAAGGAGATCAATG-3′; #720/#721, 5′-TGG-CAGGGGAGGACGCCG-3′/5′-CTGGCTCAACCAATGAGG-3′; #693/#686, 5′-CATAATGCACTTGCCTAC-3′/5′-GAGTACATACCGCCA-AGA-3′; #715/#716, 5′-GCCTGGGCACGTTGTCGGAG-3′/5′-GTC-TATACCGAAGATTTGGTG-3′; #713/#765, 5′-AGGTATTCAGGG-ATGTCTTGG-3′/5′-CGAGGAAGTTCCGTTCGCTGC-3′; #713/#768, 5′-AGGTATTCAGGGATGTCTTGG-3′/5′-GTGGGCAAGGGAGGG-GAAC-3′; #713/#712, 5′-AGGTATTCAGGGATGTCTTGG-3′/5′-ATT-CCATCCGATCCAAGTTCG-3′). For the RT reaction described in Figure 6D the primer #714, 5′-GGTCGATACTGGTAGATCGAC-3′, was used.

Plasmid constructions

The largest *Not*I fragment of cosmid X25:A8 was cloned into the *Not*I site of pMOcosX (Orbach, 1994) to make pX25L. pEK13, pEK14 and pEK15 were constructed by cloning the 8 kb *Eag*I–*Eag*I fragment, the 4 kb *Eag*I–*Eag*I fragment or the 12 kb *Not*I–*Bsp*120I fragment from cosmid X25:A8, respectively, into *Not*I-digested pMOcosX. pEK16-1 and pEK17 were constructed by cloning the 4.3 and 4.8 kb *Hin*dIII fragments from λ11–1, respectively, into the *Hin*dIII site of pCSN43 (Staben *et al*., 1989). pEK19 was constructed by cloning the 4.9 kb *Not*I–*Eco*RI fragment from cosmid X25:A8 into pBluescriptII SK(+) (Stratagene, La Jolla, CA) digested with *Not*I and *Eco*RI. pEK21 was constructed by cloning the 3.1 kb *Bgl*II–*Hin*dIII fragment from pEK15 between the *Bam*HI and *Hin*dIII sites of pBR322. To construct pEK22, a 2.2 kb PCR fragment generated from pEK19 [using primers #694 (5′-CTGCCTTCAAGTTCGTCA-3′) and #707 (5′-AACCTGCATGC-TATGACCATGATTACGC-3′) and *Pfu* DNA polymerase] was digested with *Sph*I and *Kpn*I to yield a 1.7 kb fragment (containing the 3′ part of the *dim-2* ORF) that was ligated with the 1.6 kb *Kpn*I–*Mlu*I fragment of pCSN43 (containing the *hph* gene), and inserted into pEK21 digested with *Sph*I and *Mlu*I. To build pEK24, the 0.9 kb *Eco*RI–*Xma*I fragment (containing the *qa-2* promoter) from pMYX2 (Campbell *et al*., 1994) was ligated to the 0.9 kb *Bgl*II–*Ngo*MIV fragment from pEK14 (starting 94 nucleotides upstream of the ATG codon in the *dim-2* ORF) and the combined fragment was inserted into pEK20 digested with *Bgl*II and *Eco*RI. pEK20 was constructed by cloning the 4.9 kb *Not*I–*Eco*RI fragment from pEK19 into pBM61 (Margolin *et al*., 1997) digested with *Not*I and *Eco*RI. pEK27 was constructed by cloning the 2134 bp, *dim-2*-containing *Sph*I–*Xma*I fragment from pEK19 into pMTL21 (Chambers *et al*., 1988) digested with *Sph*I and *Xma*I. pEK28-33 was built from pEK27 by PCR with *Pfu* polymerase using primers #777 (5′-CCACTGGATGAAGTCGCTC-3′) and #778 (5′-AAGGCTTTC-CAGAACTGGC-3′) and ligation. The product of the ligation reaction was digested with *Dpn*I to destroy traces of the pEK27 template. A 2107 bp *Sph*I–*Xma*I fragment of pEK28-33 was sequenced to verify the presence of the 27 bp deletion (corresponding to the ASKAHGKSK amino acid sequence of Dim-2) and the absence of mutations in the amplified sequence. pEK30–5 was constructed by cloning the 2107 bp *Sph*I–*Xma*I fragment of pEK28–33 into pEK24 digested with *Sph*I and *Xma*I, and the construct verified by DNA sequencing. pEK31 was constructed by PCR from pEK27 using Pfu polymerase and primers M13F (5′-GTAAAACGACGGCCAGT-3′) and #782 (5′-GTACCCGGG-AGCAGGGCTACC-3′; the underlined sequence, which corresponds to the complement of an alanine codon, replaces a cysteine codon in the wild-type *dim-2* sequence). The PCR product was digested with *Xma*I and *Sph*I and inserted between sites for these enzymes in pMTL21. pEK32 was constructed by cloning the 2134 bp *Sph*I–*Xma*I fragment from pEK31 into pEK24 digested with *Sph*I and *Xma*I, and confirmed by sequencing. pEK34 was constructed by PCR overlap extension mutagenesis, using the primers #687 (5′-TCGAGGACGCCATACTTG-3′), #856 (5′-CGCATG-GGGCGTCAAAGGCTTTCC-3′), #728 (5′-TACCGCCTCCGCAGT-CAAC-3′) and #857 (5′-GGAAAGCCTTTGACGCCCCATGCG-3′), to replace the lysine at position 333 of Dim-2 with an alanine nine (the codon is underlined). The PCR fragments obtained with primer pairs #687/#856 and #728/#857 were used as templates in a PCR step using primers #728 and #687. The final PCR product was digested with *Xma*I and *Sph*I and inserted between sites for these enzymes in pEK24. The 2.1 kb *Sph*I–*Xma*I fragment of pEK32 and pEK34 was sequenced to verify the intended mutations and to rule out the occurrence of chance mutations in the amplified sequences.

DNA sequence analyses

The 4.8 kb *dim-2* region (nucleotides 690–5500 in Figure 6) was sequenced on both strands from plasmids pEK15 and pEK19 on an ABI 377 automated sequencer at the University of Oregon Biotech Facility. The cDNA clone λ12-1-1 (Figure 6A) was also sequenced. To characterize the *dim-2*^{1} allele of strain N1257, PCR fragments were amplified from genomic DNA and sequenced directly. Similarly, genomic DNA of strain N1912 was amplified (primers #691, #728) and sequenced to verify the presence of the alanine codon. To safeguard against mutations potentially introduced by PCR, the products of five independent 20 µl PCR reactions were pooled for each sequencing reaction. The PCR products were fractionated in 1% agarose and gel purified with a QIAquick Gel Extraction Kit (QIAGEN). Both strands of DNA were sequenced in the PCR fragment found to carry the mutation (primers: 5′-CATAATGCACTTGCCTAC-3′ and 5′-TCTGGAAGG-AGATCAATG-3′). Computer-based sequence analyses were carried out using MacVector/AssemblyLIGN and GCG software (Oxford Molecular). The wild-type *dim-2* sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession No. AF348971.

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