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A novel DNA modification by sulphur

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Summary

Streptomyces lividans **has a novel DNA modification, which sensitises its DNA to degradation during electrophoresis (the Dnd phenotype). The entire gene cluster (*dnd*) involved in this modification was localized on an 8 kb DNA fragment and was expressed in a *S. lividans* deletion mutant (*dnd*) and in several heterologous hosts. Disruption of the *dnd* locus abolishes the Dnd phenotype, and gain of the *dnd* locus conferred the Dnd phenotype respectively. Extensive analysis of the *dnd* gene cluster revealed five open reading frames, whose hypothetic functions suggested an incorporation of sulphur or a sulphur-containing substance into *S. lividans* genome, yet in an unknown manner. The Dnd phenotype was also discovered to exist in DNA of widespread bacterial species of variable origin and diverse habitat. Similarly organized gene clusters were found in several bacterial genomes representing different genera and in eDNA of marine organisms, suggesting such modification as a widespread phenomenon. A coincidence between the Dnd phenotype and DNA modification by sulphur was demonstrated to occur in several representative bacterial genomes by the *in vivo* ^{35}S-labelling experiments.**

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Introduction

Methylation is the most common covalent modification of DNA so far reported (Luria and Human, 1952; Pradhan *et al*., 1999), although some bacteriophages modify their DNA in other ways (Hattman, 1979; 1980; Swinton *et al*., 1983). An unusual and seemingly novel DNA modification was discovered in the Gram-positive bacterium *Streptomyces lividans* 66 (Zhou *et al*., 1988; Boybek *et al*., 1998), a commonly used host strain for gene cloning in *Streptomyces* (Kieser *et al*., 1982; Hopwood *et al*., 1983). This modification results in the degradation of DNA *in vitro* by oxidative, double-stranded, site-specific cleavage during normal (Zhou *et al*., 1988; Boybek *et al*., 1998; Dyson and Evans, 1998) and pulsed-field gel electrophoresis (Kieser *et al*., 1992; Zhou *et al*., 1994). This phenotype is named Dnd (for DNA degradation). Such double-stranded scission at modification sites was proved to be a peracid-mediated, oxidative and amine-catalysed reaction (Ray *et al*., 1992; 1995). The DNA modification is most likely on a base, at G-specific sites, possibly after normal DNA is synthesized (Boybek *et al*., 1998). DNA isolated from a modification-deficient deletion mutant, ZX1, is no longer degraded during electrophoresis (Zhou *et al*., 1988; 1994). The Dnd phenotype, implying a similar modification, was also discovered in *Streptomyces avermitilis*, producer of the commercially important antihelminthic antibiotics, the avermectins (Kieser *et al*., 1992; Evans *et al*., 1994). However, such a modification was not observed in *Streptomyces coelicolor* A3(2), whose genome is very similar to that of *S. lividans* even at the sequence level (Kieser *et al*., 1992; Leblond *et al*., 1993).

Here we demonstrate that this unusual DNA modification is determined by a cluster of five genes (*dnd* cluster), which was cloned and sequenced. The loss or gain of the Dnd phenotype diagnostic for the modification coincided well with the disruption or acquisition of the *dnd* cluster. Bioinformatic analysis of the complete *dnd* gene cluster allowed us to propose a biochemical mechanism involving the possible incorporation of sulphur or a sulphur-containing substance into DNA, which was subsequently proven by the *in vivo* ^{35}S-labelling experiments, although the exact chemical structure of the specifically modified base remains undetermined. The comparative searches for the *dnd* homologues and/or the Dnd phenotype, in turn, led to the discovery of a similar modification system

in many unrelated bacteria representing different genera, and in eDNA of marine organisms.

Results

DNA modification associated with the Dnd phenotype is governed by the dnd *locus*

ZX1, a *dnd*⁻ mutant of *S. lividans* 66, has an approximately 90 kb chromosomal deletion (Zhou *et al*., 2004) that includes the putative DNA modification (*dnd*) gene(s). The *dnd* locus was mapped to one (16C3) of a set of 13 ordered cosmids (Zhou *et al*., 2004) covering the deleted region, by integrating the cosmids, whenever overlaps made it possible, into the ZX1 chromosome via homologous recombination and looking for complementation of the Dnd⁻ phenotype. Total DNA of a cosmid-16C3-derived strain (ZX1::16C3) was degraded during electrophoresis (Fig. 1, lane 3), like wild-type *S. lividans* DNA (Fig. 1, lane 2), while that of a derivative carrying another cosmid, ZX1::17G7 (Fig. 1, lane 1), was not, indicating that 16C3 contained genes for the specific DNA modification associated with the Dnd phenotype

A more precise localization of the *dnd* gene(s) on 16C3, which carries about 40 kb of chromosomal DNA, was achieved by subcloning and tests of complementation of the Dnd⁻ phenotype by specific regions of the cosmid. MboI fragments of different sizes were cloned into the BamHI site of the vector pSET152 (Bierman *et al*., 1992), containing the *attP* site of phage ΦC31, which integrates into the host *attB* site. The smallest pSET152-derived plasmid (pHZ1904) that complemented the Dnd⁻ phenotype of ZX1 DNA had an insert of 8026 bp (Fig. 1).

The dnd *locus consists of five open reading frames (ORFs)*

The 8026 bp region of pHZ1904, presumed to carry the entire *dnd* gene cluster, was sequenced (Gene bank Accession number DQ075322), and five ORFs were identified (designated *dndA–E*, Fig. 1). The G+C content of the cluster is 65.65%, somewhat lower than the average for *S. coelicolor* of 72.12% G+C (Bentley *et al*., 2002), while the codon usage of *dndA–E* is typical of *Streptomyces*. *dndA* and *dndB–E* are divergently transcribed, so that divergent promoters are likely to lie in a 76 bp region between the translational initiation codons of *dndA* and *dndB*. *dndB–E* most likely constitute an operon as the 3′ end of *dndB* and the 5′ end of *dndC* overlap by 4 bp at nucleotides 3605 and 3608 (ATGA), the initiation codon (ATG) of *dndD* precedes the 3′ end of *dnd*C by 12 bp (5088-ATGCACCTGCATAA-5098), and the initiation codon of *dndE* (ATG) is 9 bp upstream of the stop codon of *dndD* (ATGCCGTCTGA).

*dnd*A is predicted to encode a protein of 380 amino acids with a molecular weight of 41 kDa and close sequence resemblance (46% identity, 64% similarity) to two of the most central proteins for Fe-S cluster formation. These are IscS (*i*ron *s*ulphur *c*luster) proteins (Fig. 2), involved in the biosynthesis of 4-thiouridine, and the closely related NifS (*ni*trogen *f*ixation) proteins of nitrogen-fixing bacteria (e.g. *Klebsiella pneumoniae* and *Rhodobacter capsulatus*). The functions of IscS and NifS are related in that they provide the sulphur via an L-cysteine desulphurase activity. All of these proteins, including DndA, have a conserved pyridoxal phosphate-binding motif.

*dnd*B is predicted to encode a protein of 376 residues with a molecular weight of 37 kDa. The gene product is

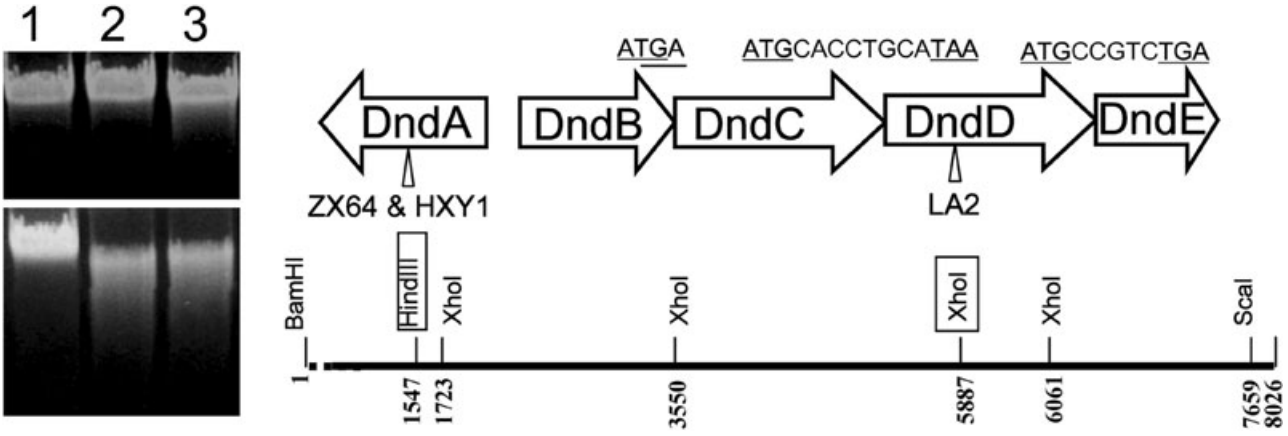

Fig. 1. Left bottom. Complementation of the Dnd phenotype of ZX1 by 16C3 (lane 2) but not 17G7 (1). *S. lividans* 1326 (3) was used as a positive control.
Left top. All of the DNA samples used were proven to be good in gel electrophoresis buffer with added thiourea (Ray *et al*., 1992; 1995).
Right. Five genes involved in the Dnd phenotype of *S. lividans*. Vertical arrowheads indicate the insertion points in *dndA* and *dndD* after gene disruptions at the boxed restriction sites of the complete *dnd* gene cluster (bottom). The DNA sequences between adjacent genes in the putative *dndBCDE* operon are shown at the top, with start and stop codons underlined.

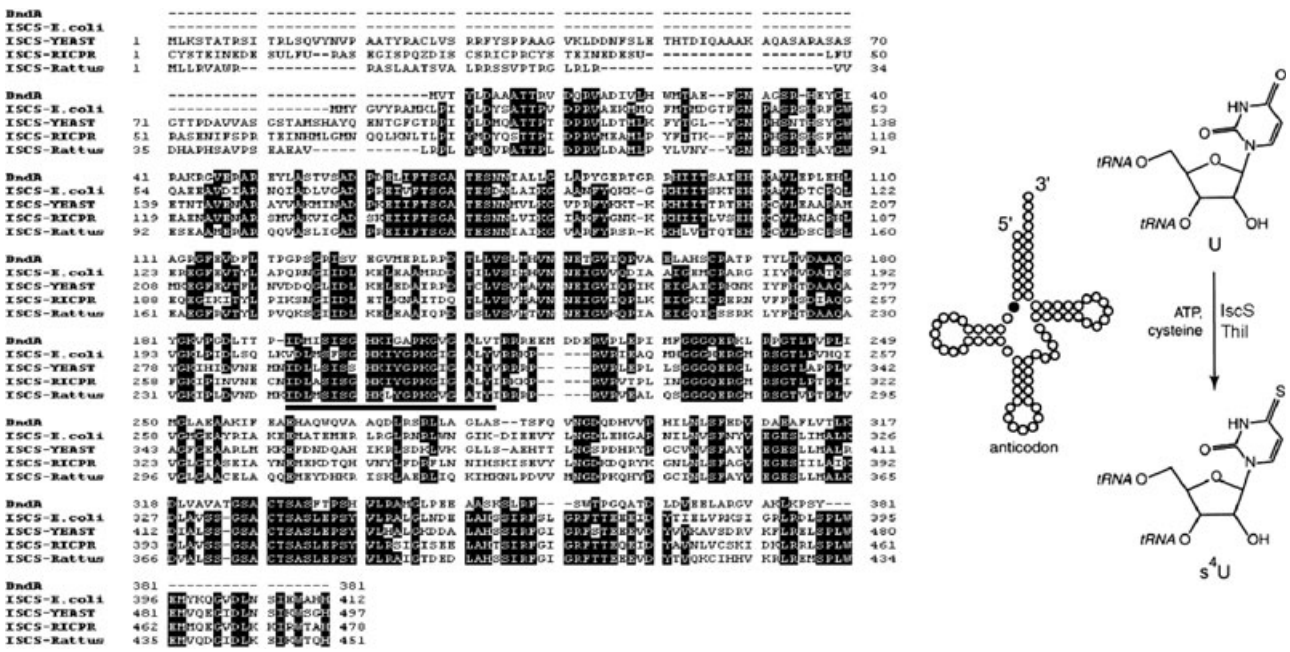

Fig. 2. Left. Alignment of IscS homologues. The deduced protein sequence of *DndA* from *S. lividans* resembles IscS proteins and their homologues from prokaryotic and eukaryotic microorganisms. Included in the alignments are IscS homologues from *E. coli* (E. coli), *Saccharomyces cerevisiae* (YEAST), *Rickettsia prowazekii* (RICPR), and *Rattus norvegicus* (Rattus) respectively. All the proteins, including *DndA*, contain a pyridoxal phosphate–binding motif (underlined).
Right. Formation of 4-thiouridine catalysed by IscS and ThiI at uridine-8 of certain bacterial tRNA (shown as a filled circle) in the presence of ATP and cysteine.

18% identical and 37% similar to an ATPase involved in DNA repair from *Clostridium thermocellum* ATCC 27405, and showed significant amino acid sequence homology to a group of putative transcriptional regulators. A run of 152 residues is 24% identical and 36% similar to the substrate-binding protein of an ABC transporter of *Streptococcus pneumoniae* TIGE4.

DndC (a putative protein of 498 residues) showed significant alignments with two enzymes: 18% identity (35% similarity) to an ATP sulphurylase in *Klebsiella aerogenes* and a series of such enzymes in many other bacteria, including *E. coli*; and 21% identity (35% similarity) to a phosphoadenosine phosphosulphate (PAPS) reductase-related protein in *Pyrococcus abyssi* and the same family of proteins from many other bacteria. Both enzymes are involved in sulphate reduction for the biosynthesis of sulphur-containing compounds such as cysteine by many microorganisms. ATP sulphurylase is required for activation of inorganic sulphate before it can be reduced, producing the high-energy species APS (adenylylsulphate) and pyrophosphate, while PAPS reductase is used to reduce PAPS to sulphide. PAPS reductase is part of the adenine nucleotide alpha hydrolase superfamily that includes N-type ATP PPases and ATP sulphurylases. Accordingly, we found a specific 'P-loop' (42-SGGKDS-47), the so called PP motif (Bork and Koonin, 1994), which interacts with the phosphate of AMP, as commonly found in the PPi synthetase family, with a consensus sequence SGGXD(S/T) critical for enzymes that catalyse adenylation.

dndD is predicted to encode a protein of 663 residues with significant homology (23% identity, 45% similarity) with the ABC transporter ATP-binding protein of *Fusobacterium nucleatum* ssp. *nucleatum* ATCC 25586. The ATP-binding cassette (ABC) is a highly conserved protein domain of about 215 residues that plays a role in transducing the energy of ATP hydrolysis to a wide variety of physiological processes in all eukaryotic, archaebacterial and eubacterial species studied. Accordingly, an ATP/GTP-binding Walker A motif (35-GLNGCGKT-42) and a good ABC transporter family signature (556-LSAGERQL-LAISLLW-570) were detected.

DndE, a small putative protein of 126 residues, has 46% identity and 61% similarity to phosphoribosylaminoimidazole carboxylase (NCAIR synthetase) from *Anabaena variabilis* ATCC 29413 (Fig. 3). Many such enzymes may become part of a larger enzyme complex by coupling with both ATPase and NCAIR synthetase (Nakamura *et al*., 2002).

Disruption of the dnd *locus abolishes the DNA modification*

To address the relevance of the *dnd* genes in the Dnd

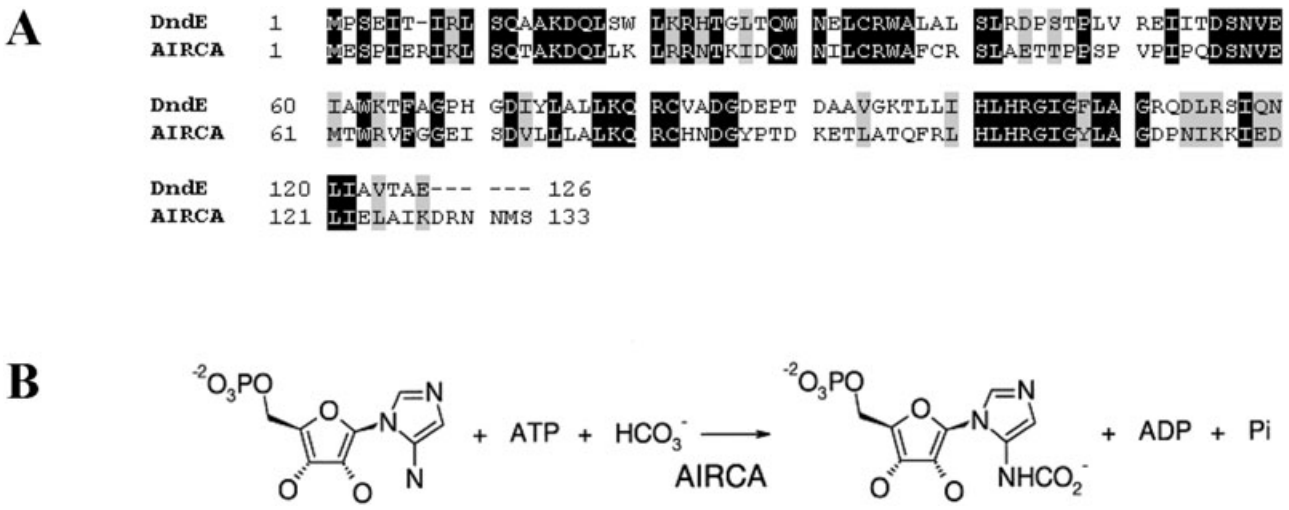

Fig. 3. Alignments of DndE with phosphoribosylaminoimidazole carboxylase (NCAIR synthetase) from *Anabaena variabilis* ATCC 29413 (A), which is known to usually act at a condensing carboxylation step, in purine biosynthesis (B).

phenotype, mutant strains were engineered by inserting the spectinomycin/streptomycin resistance cassette (*aadA*) into two of the five ORFs in the genome (Fig. 1).

Total DNA isolated from HXY1, ZX64 and LA2 (Fig. 1) was not degraded during conventional electrophoresis or PFGE, indicating that both of the insertion sites lie in essential *dnd* genes. Moreover, the mutations in *dnd*A (HXY1 and ZX64) and *dndD* (LA2) could be complemented by re-introducing pHZ825 (carrying the entire *dnd* cluster) into the genome of each mutant strain, to regain the Dnd phenotype. Furthermore, neither of the 12 kb EcoRI fragments with *spc/str* insertions used to generate HXY1, ZX64 (pHZ828) and LA2 (pHZ895) could confer the Dnd phenotype on ZX1, the mutant with a complete deletion of the *dnd* gene cluster, when cloned into the EcoRI site of pSET152, introduced by transformation and integrated into the ZX1 genome.

Gain of the dnd *locus conferred the DNA modification*

The correlation of the Dnd phenotype with the specific DNA modification was further demonstrated by gain of Dnd functions. pHZ1904 carrying the complete *dnd* gene cluster could be introduced into *Streptomyces nanchangensis* NS3226 (ZX67, Fig. 4), *Streptomyces parvulus* ATCC12434 (ZX68, Fig. 4) and *Micromonospora* sp. 40027 (Li *et al*., 2003), all with DNA that is stable during electrophoresis and lacks DNA modification. Significantly, upon integration, DNA of all three species became highly unstable (Fig. 4, Li *et al*., 2003), like DNA of wild-type *S. lividans* 66. Furthermore, the *dnd* deletion mutant strain ZX1 regained its Dnd phenotype when the entire *dnd* gene cluster carried on pSET152 was integrated into its chromosome (not shown).

Such DNA modification seems to be widespread

Correlation of the Dnd phenotype with such DNA modification prompted us to look for other organisms that might have a similar modification system. A first attempt was made by analysing the DNA of selected microbes, plants and animals but only the DNA of *Mycobacterium smegmatis* and *Streptomyces acrimycini* 2236 showed the Dnd phenotype among more than 50 samples tested (not

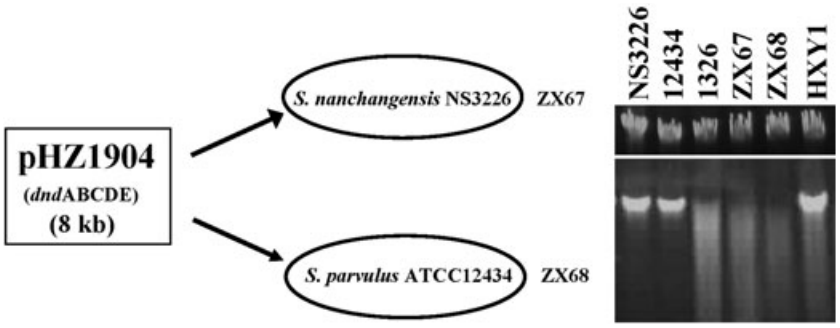

Fig. 4. Dnd phenotypes associated with the loss or gain of *dnd* function carried on pHZ1904. Loss of DndA function (HXY1 in Fig. 1) from its progenitor 1326 makes its DNA stable during electrophoresis, while addition of *dndA–E* on pHZ1904 in *S. nanchangensis* NS3226 (ZX67) and *S. parvulus* ATCC12434 (ZX68), whose wild-type DNA were stable, paralleled the Dnd phenotype, as shown (right bottom). All of the DNA samples used (right top) were proven to be good in gel electrophoresis buffer with added thiourea (Ray *et al*., 1992; 1995).

shown). A search of the literature showed that *S. avermitilis* (Evans *et al*., 1994), producer of the commercially important antihelminthic antibiotics, the avermectins, more than 50 strains of *Pseudomonas aeruginosa* used in a DNA-based typing experiment (Romling and Tummler, 2000), two *Salmonella enterica* serovar Livingstone and two serovar Cerro isolates from a commercial egg-producing farm (Murase *et al*., 2004), eight strains of *Salmonella* serovar Ohio and nine strains of *Salmonella* serovar Newport, two enterohaemorrhagic *Escherichia coli* non-O157:H7 strains (Koort *et al*., 2002), 66 Gram-negative bacteria, covering seven different species from 11 medical centres (including 22 *E. coli,* four *K. pneumoniae,* two *Enterobacter cloacae,* 12 *Serratia marcescens,* 17 *P. aeruginosa*, five *Acinetobacter* spp. and four *Salmonella* spp.) (Silbert *et al*., 2003), isolates of *Clostridium difficile* (Corkill *et al*., 2000), *Clostridium botulinum* (Hielm *et al*., 1998) and *Vibrio parahaemolyticus* (Marshall *et al*., 1999), might all have a similar system because of a widely detected Dnd phenotype, which in many cases had been interpreted as due to a contaminating DNase. Most remarkably, the Dnd phenotype was recently found in almost 50% of *Mycobacterium abscessus* isolates (69 in total) by pulsed-field gel electrophoresis (Zhang *et al*., 2004). When two isolates of *S. enterica* serovar ssp. *enterica* Livingstone and two isolates of serovar Cerro were tested under normal agarose gel electrophoresis, their DNA was found to be degraded (Fig. 5).

When the *dnd* genes were used for BLAST searches against the protein and genome databases, strong similarity was obtained to some protein sequences in microbial genomes (Fig. 6). Apart from homologies detected between DndA,C,D and their counterpart proteins with known functions, the gene101 protein (SpfB in Fig. 6) of *Pseudomonas fluorescens* PfO-1 exhibited remarkable end-to-end identity (52%) and similarity (70%) to DndB, and the gene 104 protein (SpfE in Fig. 6) exhibited a significant end-to-end identity (26%) and similarity (45%) to DndE (Fig. 6). Strong end-to-end resemblances were also detected between DndE and Npun3700 (SnpE in Fig. 6) in *Nostoc punctiforme* (47% identity, 62% similarity), alr4922 (SpcE in Fig. 6) in *Nostoc* PCC7120 (45% identity, 60% similarity), and Tery 1753 in *Trichodesmium erythraeum* IMS101 (48% identity, 61% similarity), along with strong homologies between *dndA,C,D* and corresponding genes in these genomes. Good alignments for DndB homologues were seen only when the *spc* and *snp* clusters were aligned independently of the other three clusters. (A regional strong resemblance was only detected between DndB and alr2541 (SpcB in Fig. 6) in *Nostoc* PCC7120 (28% identity, 45% similarity) but not in the other two strains.) The genes homologous to DndC,D,E are closely clustered in *N. punctiforme* and *Nostoc* PCC7120, but not in *T. erythraeum* IMS101.

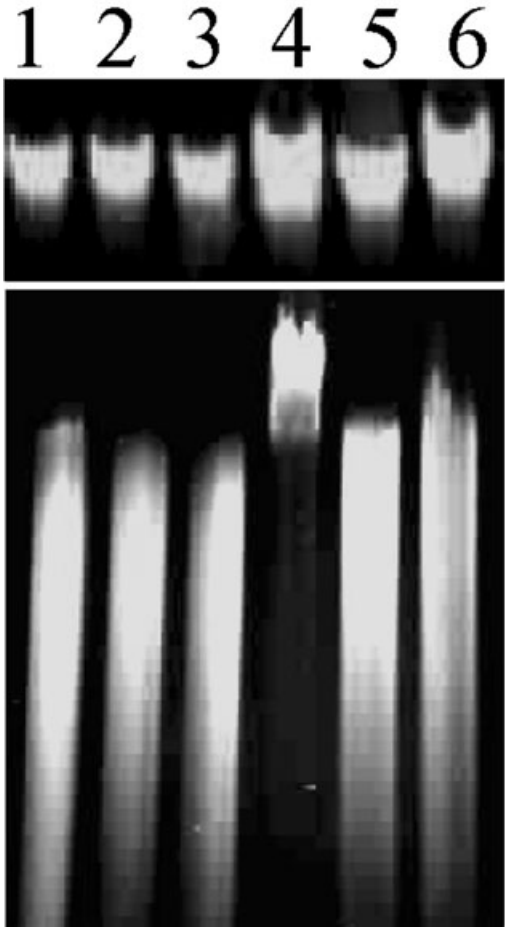

Fig. 5. Dnd phenotype displayed by *Salmonella enterica* serovar Cerro isolates 87 (1) and 114 (2), and serovar Livingstone isolates 663 (4) and 821 (5), but not from serovar Cerro isolate 163 (3) of a commercial egg-producing farm (strains kindly gifted by Prof. Toshiyuki Murase), under normal agarose gel electrophoresis (bottom). All of the DNA samples were proven to be good in normal agarose gel electrophoresis buffer with added thiourea shown at the top (Ray *et al*., 1992; 1995).

Figure 6 shows a comparative map of the putative DNA modification genes of *S. lividans* 66, *S. avermitilis* NRRL8165, *P. fluorescens* PfO-1, *Nostoc* PCC7120 and *N. punctiforme*, which showed strongest resemblance to the *S. lividans* 66 *dnd* gene cluster.

Pseudomonas fluorescens PfO-1 DNA was found to be as susceptible to degradation as that of *S. lividans* 66 and *S. avermitilis* NRRL8165 under the same electrophoresis conditions, suggesting that *P. fluorescens* PfO-1 DNA might be modified in a similar manner (not shown).

Interestingly, another BLAST search against the protein databases deduced from the eDNA of the Sargasso sea described in a recent analysis (Venter *et al*., 2004) revealed extensive homologies with all five individual Dnd proteins (A–E), among which a representative gene cluster organized in a similar fashion to *dndA–E* in *S. lividans*

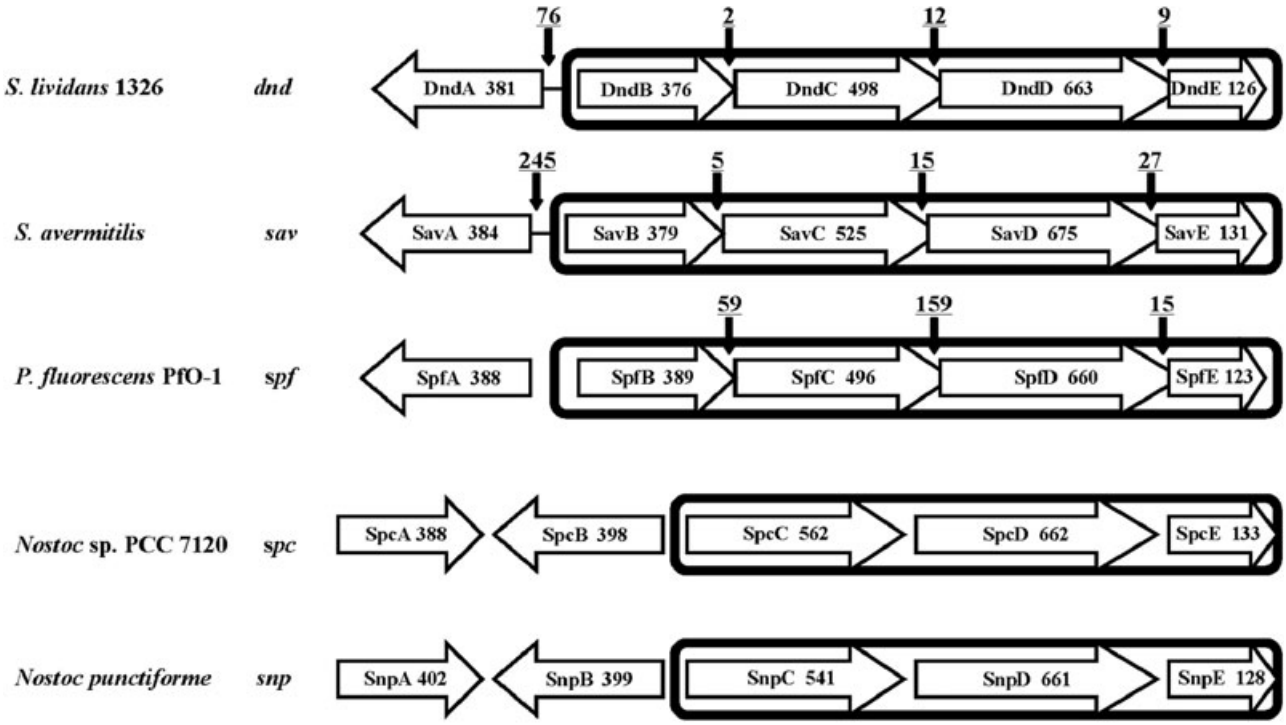

Fig. 6. Comparison of the genetic organizations of the gene clusters involved in the Dnd phenotype. The organizations of *dnd* (from *S. lividans*) and *sav* (from *S. avermitilis, svaA–E* are equivalent to SAV2933-SAV2929 in the chromosomal region 3672466-3678962 covered by cosmid number 145, Ikeda *et al*., 2003) are very similar in that they share five genes encoding proteins of similar sizes (numbers of amino acid residues inside the arrows) that are all clustered and all of the corresponding *BCDE* genes constitute putative operons. The *spf* cluster from *P. fluorescens* PfO-1 differs from *dnd* and *sav* by having *spcA* separated from the *spfBCDE* operon in the genome. The *spc* (from *Nostoc* sp. PCC7120) and *snp* (from *Nostoc punctiforme*) genes are more different in organization. The clustered genes are boxed and numbers of nucleotides between the *A* and *BCDE* operons and between adjacent genes among *BCDE* operons are indicated above the ORFs wherever applicable.

66 could be identified, although the corresponding DndA and DndC seemed to be incomplete because of their location at opposite ends of a 1370 bp sequenced contig (AACY01077015), and sequences corresponding to DndD were found in two smaller clustered proteins in a 2239 bp sequenced contig (AACY01077016). Again, counterparts of DndA and the DndBCDE operon are 96 bp apart and the genes appear to be divergently transcribed.

Sulphur was found to be incorporated into DNA of S. lividans *66,* S. avermitilis *NRRL8165, and* P. fluorescens *PfO-1*

Several *Streptomyces* strains were propagated in minimal medium containing $^{35}SO_4$, and total genomic DNA was run on an agarose gel and analysed by Southern blotting. Remarkably, ^{35}S was associated with the high molecular weight DNA of wild-type *S. lividans* (Fig. 7, lane 1). DNA of the *S. lividans dnd* deletion mutant, ZX1 (lane 2), and *S. coelicolor* (lane 3) lacking the Dnd phenotype, showed no radioactivity, as was also true for the mutants HXY1 (lane 4) and LA2 (lane 5) generated from wild-type *S. lividans* by targeted gene disruptions (Fig. 1), indicating a coincidence between S modification and the Dnd phenotype. One atom of S was calculated to be present per approximately 6000 bp, by scintillation counting of the ^{35}S-labelled DNA, in agreement with earlier estimates for the extent of the modification before it was characterized (Zhou *et al*., 1988).

Similarly, when DNA of two other representative strains, *S. avermitilis* NRRL8165 (Fig. 7, lane 6) and *P. fluorescens* PfO-1 (Fig. 7, lane 7), was analysed for S modification as described above, both were also shown to contain ^{35}S.

Discussion

Identification and characterization of a gene cluster for a novel DNA modification by S associated with the Dnd phenotype in the DNA of *S. lividans* 66, *S. avermitilis* and *P. fluorescens* PfO-1, is intriguing. This unprecedented discovery raises a number of immediate questions, among which are the exact chemical nature and the associated biological significance and incidence of the modification among diverse organisms.

Transfer RNAs of most organisms contain a variety of chemical modifications that serve to modulate their inter-

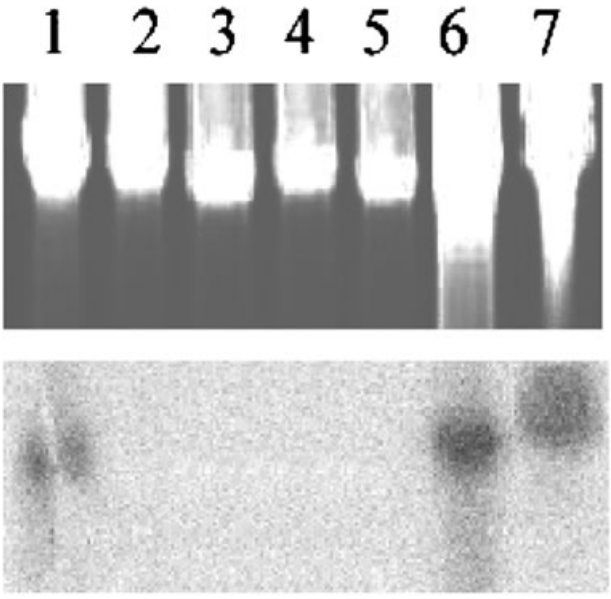

Fig. 7. Detection of S incorporation in DNA of wild-type *S. lividans* 1326 (lane 1), but not in its mutants ZX1 (2), HXY1 (4) and LA2 (5) or in *S. coelicolor* J1501 (3). S is also detected in DNA of *S. avermitilis* (6) and *Pseudomonas fluorescens* PfO-1 (7). Radioactive signals detected after Southern transfer (bottom) of the ethidium bromide-stained agarose gel (top) indicate the presence of S in the DNA.

actions with both proteins and other RNAs, such as mRNA (Lauhon and Kambampati, 2000; Mueller *et al*., 2001). Several of these modified bases contain sulphur (Ajitkumar and Cherayil, 1988). The predominant thionucleotide in *E. coli* tRNA is 4-thiouridine (s^4U), formed post-transcriptionally by modification of the nearly invariant uridine at position 8 (Fig. 2). Synthesis of s^4U requires IscS, which provides S via an L-cysteine desulphurase activity (Kambampati and Lauhon, 2000; Lauhon and Kambampati, 2000), and ThiI, a protein with adenylation and sulphurtransferase activities (Kambampati and Lauhon, 2000; Palenchar *et al*., 2000; Mueller *et al*., 2001). ThiI was originally identified as one of a series of proteins involved in thiamine biosynthesis (Taylor *et al*., 1998). IscS and ThiI alone suffice for generation of 4-thiouridine (Mueller *et al*., 2001). IscS converts cysteine into alanine and forms a persulphide group on one of its cysteines, providing a source of the persulphide group on ThiI (by transpersulphidation). Two mechanisms were proposed that account for the participation of a persulphide in 4-thiouridine generation (Mueller *et al*., 2001), both of which involve two proteins, IscS and ThiI.

The precise chemical nature and hence the biochemical pathway of the DNA S modification associated with the Dnd phenotype in *S. lividans*, which was located in a G-specific site, possibly after normal DNA is synthesized (Boybek *et al*., 1998), remains obscure. We also failed so far to obtain sufficient amounts of pure sample, using either genomic or a piece of DNA fragment originated from a *Streptomyces* plasmid pIJ101 (Zhou *et al*., 1988; Boybek *et al*., 1998), which was known to carry a preferential modification site, for chemical determination of the modified base(s), although extensive effort had been made. This could either be due to the extreme scarcity or possible instability *in vitro*, of the likely site-specifically modified base(s).

The extensive similarity of the DndA protein to IscS-homologues, and the catalytic activity of the IscS protein for the mobilization of S into tRNA in s^4U synthesis, tends to suggest its role as an L-cysteine desulphurase. The homology of DndC to subunit 2 of the sulphate adenylate transferase in the PAPS reductase family of proteins, to constitute a domain for adenylation functionality, along with the finding of an adenylation-specific P-loop, clearly agrees with the role of ThiI. Involvement of at least five proteins, more than those necessary for the generation of 4-thiouridine in tRNA modification, would suggest some additional mechanisms for the site-specific DNA modification. The modified base was unlikely to be formed by the simple substitution of a carbonyl by a thiocarbonyl group (e.g. to form 6-thioguanine), but more likely by the carboxylation of an unknown compound to a preformed S-containing base (e.g. 6-thioguanine), because one of the five putative enzymes (DndE) catalysing a putative carboxylation step, which is highly similar to phosphoribosylaminoimidazole carboxylase (NCAIR synthetase) of the purine biosynthetic pathway from *A. variabilis* ATCC 29413, was found to be necessary. Such enzyme activities need ATP, and could be coupled with an independent ATPase, or even become part of a larger enzyme with both activities. DndD, an SMC-like ATPase closely linked with DndE with a distinguishable myosin-tail consisting of a coiled-coil region and a flexible hinge, which might function as an ATP-modulated DNA cross-linker and energy generator by ATP hydrolysis, could obviously provide this. Conceivably, additional functionality would be required for the determination of sequence specificity, within GC-rich regions with potential to form complex secondary structures (Boybek *et al*., 1998). This is likely encoded by *dndB*, whose product showed homology to an ATPase involved in DNA repair from *C. thermocellum* ATCC 27405, and to a group of transcriptional regulators.

The above implications, especially the fact that the modification system involves at least five proteins, and counterpart proteins obviously equivalent to IscS and ThiI for the tRNA modification to form the 4-thiouridine (s^4U) were present, apparently suggested that sulphur or a sulphur-containing substance may have been incorporated into the specific site(s) of the DNA, and led subsequently to a direct proof by the ^{35}S-labelling experiment.

Similar DNA S modification reflected by the presence of the identical gene clusters (Fig. 6) and diagnostic Dnd

phenotype observed in many other Gram-positive and Gram-negative bacteria from various genera, including isolates of *P. fluorescens, P. aeruginosa*, *E. coli, K. pneumoniae, E. cloacae*, *S. marcescens*, *Acinetobacter* spp. *Salmonella* spp., *C. difficile*, *C. botulinum*, *M. smegmatis, M. abscessus, S. acrimycini* 2236 and *V. parahaemolyticus*, etc., suggests that such systems are more widespread, at least in bacteria than had hitherto been realized. We observed a similar level of the Dnd phenotype in *S. lividans* 66, *S. avermitilis, S. acrimycini* 2236 and *P. fluorescens* PfO-1, but we also found much reduced DNA degradation in *M. smegmatis,* two isolates of *S. enterica* serovar Livingstone and two isolates of serovar Cerro, and heterologous expression of the *dnd* gene cluster in *Micromonospora* sp. 10027 gave rise to much less severe DNA degradation *in vitro* than in its native host, *S. lividans* 66 (Li *et al*., 2003). Thus, specific modification sites in different organisms could vary dramatically, at least partly due to variable G+C content. In addition, the abundant presence of *dnd* homologues in eDNA from the Sargasso sea samples may suggest a more widespread occurrence of DNA S modification in marine organisms. Obviously, these findings will begin to provide a basis for the understanding of such well-known but puzzling phenomenon associated with the widely observed chronic instability, at molecular level in these organisms.

Streptomyces coelicolor, a very close relative of *S. lividans*, with near 100% sequence identity among shared genes, obviously lacks the Dnd system and the *dnd* gene cluster. The G+C content of the 8026 bp *dnd* gene cluster (65.65%), lower than the average for *S. coelicolor* of 72.12% (Bentley *et al*., 2002), but close to that of *Pseudomonas* and *Mycobacterium,* may suggest that the Dnd system did not originate from *Streptomyces*, and could thus be anticipated to be more widespread in its progenitors. The very recent demonstration of a genomic island in *S. lividans* 66, carrying the *dnd* gene cluster (Zhou *et al*., 2004), strongly supports this presumption, and also implies that the Dnd system could be mobile among diverse organisms, although the evolutionary consequences in different organisms are not at all clear at present. We suggest that continued biological studies on the *dnd* system should not be confined to *Streptomyces*, but rather on a wider spectrum of microbial systems in relation to phenotypes such as pathogenecity, metabolism, growth rate, in diverse bacteria known to display the Dnd phenotype, for uncovering new biological functions. It is thus anticipated that molecular study of such systems from Gram-negative bacteria like *Salmonella*, apart from the continued effort on the mutational and biochemical analysis for the association of the Dnd system with specific biological phenotypes in *Streptomyces*, will be of great significance in widening our understanding in this fascinating new field. Some aspects of these works are now in progress.

Experimental procedures

Bacterial strains and plasmids

These are described in Table 1.

General methods and techniques

General growth media and conditions for *E. coli* and *Streptomyces* strains, standard methods for handling *E. coli* and *Streptomyces in vivo* and *in vitro*, such as preparation of plasmid and chromosome DNAs, construction of a *Streptomyces* genomic library in *E. coli*, transformation and Southern hybridization, etc., were as described in (Kieser *et al*., 2000) and (Sambrook *et al*., 1989) unless otherwise stated. pSET152 derivatives were integrated into *S. nanchangensis* NS3226 and *S. parvulus* ATCC 12434 after conjugation from *E. coli*. The cosmids constructed using SuperCos1 were mapped using a starting cosmid for walking in both directions using non-radioactive T3 and T7 riboprobes of the insert ends to identify overlapping clones. Subsequent rounds of hybridization used probes generated from overlapping cosmids chosen from the previous step of hybridization. DNA sequencing was carried out using ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kits from PE Applied Biosystems. DNA fragments were subcloned into pBluescriptII SK (+) followed by unidirectional subcloning using the Erase-a-Base system (Promega). The double-stranded plasmid DNA used as template was purified by PEG8000 precipitation before sequencing. About 200–400 ng of template DNA was mixed with 4 μl of Terminator Ready Reaction mix and appropriate primer to bring the total volume to 20 μl with deionized water. The sequencing reaction mixes were subjected to cycle sequencing on the DNA Thermal Cycle (polymerase chain reaction) machine before running the sequencing gel using an ABI autosequencer 377.

Tests of Dnd phenotype

Methods described by (Zhou *et al.,* 1988) or (Dyson and Evans, 1998) and (Ray *et al*., 1992; 1995) were used. Total DNA of wild-type *S. lividans* 1326 (Dnd+) was used as positive control and that of mutant ZX1 (Dnd^-) as negative control.

Analysis of ^{35}S-labelled DNA in vivo

Total DNA was isolated from mycelium of *S. lividans* 66, *S. avermitilis* and *P. fluorescens* PfO-1 after growth in minimal medium (Kieser *et al*., 2000) containing 36 nM Na_2SO_4 and 10 nM ^{35}S-labelled Na_2SO_4 for 3 days. The total DNA was treated with proteinase K and RNase to remove proteins that contain S and RNA, fractionated on an agarose gel and blotted to Hybond N nylon membrane for Southern hybridization. A Fujifilm FLA3000 phosphoimager was used to detect radioactivity of the Southern blots and a Wallac 1450 Microbeta Trilux system was used for liquid scintillation counting.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Characteristics[a]	Source or reference
S. lividans 66		
1326	Prototrophic, SLP2$^+$ SLP3$^+$	Feitelson and Hopwood, (1983)
JT46	*str*-6 *pro*-2	Chen *et al*. (1987); Kieser *et al*. (1989)
ZX1	JT46 derivative, selected for its DNA stability during electrophoresis, *dnd*	Zhou *et al*. (1988)
ZX7	ZX1 derivative with better sporulation, *dnd*	Hu *et al*. (1994)
ZX64	JT46 derivative, *dndA* interruption mutant	Fig. 1
HXY1	1326 derivative, *dndA* interruption mutant	Fig. 1
LA2	1326 derivative, *dndD* interruption mutant	Fig. 1
S. coelicolor A3(2)		
J1501	*hisA1 uraA1 strA pgl* SCP1$^-$ SCP2$^-$	Chater *et al*. (1982)
S. nanchangensis		
NS3226	Wild-type producer of nanchangmycin and meilingmycin	Ouyang *et al*. (1984)
S. parvulus		
ATCC12434	Wild-type	Hopwood *et al*. (1985)
Micromonospora sp.		
40027	Wild-type producer of fortimycin A	Ma *et al*. (1986)
S. avermitilis		
NRRL8165	Wild-type producer of avermectins	Evans *et al*. (1994)
P. fluorescens		
PfO-1	Wild-type, Dnd$^+$	Gift from Dr Eduardo Robleto
Salmonella		
87	Serotype Cerro, Dnd$^+$	Gift from Dr Toshiyuki Murase
114	Serotype Cerro, Dnd$^+$	Gift from Dr Toshiyuki Murase
163	Serotype Cerro, Dnd$^-$	Gift from Dr Toshiyuki Murase
663	Serotype Livingstone, Dnd$^+$	Gift from Dr Toshiyuki Murase
821	Serotype Livingstone, Dnd$^+$	Gift from Dr Toshiyuki Murase
E. coli K-12		
DH5α	F$^-$ *rec*A *lac*Z ΔM15	Hanahan, (1983)
ET12567	*dam dcm hsdS*	MacNeil *et al*. (1992)
LE392	*su4pE4 supF58 hsdR514*, used for infection with *in vitro*-packaged cosmids	Borck *et al*. (1976)
Plasmids		
pSET152	aac(3) IV *lacZ rep^{puc} att$^{\Phi C31}$ ori T*	Bierman *et al*. (1992)
Supercos 1	*bla neo rep^{puc} cos*	Evans *et al*. (1989)
16C3	Supercos 1 derived cosmid carrying *dnd* gene cluster	Zhou *et al*. (2004)
pHZ825	SuperCos1 derivative carrying a 12 kb EcoRI fragment including the 8026 bp *dnd* gene cluster (Fig. 1)	This work
pHZ828	*aadA* inserted into the unique HindIII site of the 8026 bp *dnd* gene cluster (Fig. 1) in pHZ825	This work
PHZ895	*aadA* inserted into one XhoI site of the 8026 bp *dnd* gene cluster (Fig. 1) in pHZ825	This work
pHZ1904	pSET152 derivative carrying 8 kb *dnd* gene cluster	This work
pBluescript II SK(+)	Vector for DNA sequencing, *bla*	Alting-Mees and Short, (1989)

a. *oriT*, origin of transfer of plasmid RK2; *tsr*, thiostrepton resistance gene; *aadA*, spectinomycin/streptomycin resistance cassette. Dnd, DNA degradation phenotype; *pgl*, phage growth limitation.

Engineering of dnd mutants

pHZ825, a SuperCos1 (Evans *et al*., 1989) derivative carrying a 12 kb EcoRI fragment including the 8026 bp *dnd* gene cluster (Fig. 1) in the corresponding site, was used as an intermediate vector. Two suicide plasmids, pHZ828 and pHZ895, were generated from pHZ825 with the *spc/str* cassette inserted either into the unique HindIII site in *dndA* or into XhoI site 5887 in *dndD.* They were used to generate the strains ZX64 and HXY1 or LA2 respectively (Fig. 1). Gene disruptants occurring by double crossing over were obtained after pHZ828 and pHZ895 were introduced into *S. lividans*, with selection for spectinomycin/streptomycin resistance and screening for thiostrepton sensitivity. The mutant strains were confirmed by Southern hybridization.

Bioinformatic analysis

Frame analysis was carried out using Frameplot (Ishikawa and Hotta, 1999). PSI and PHI-BLAST (Altschul *et al*., 1997) were used for BLAST searches. Superfamily searches were done using SUPERFAMILY (Gough *et al*., 2001) and coiled-coil structure was analysed using COILS (Lupas *et al*., 1991). http://motif.genome.jp was used for MOTIF searches.

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