The *Bacillus subtilis* genes for ribonucleotide reductase are similar to the genes for the second class I NrdE/NrdF enzymes of *Enterobacteriaceae*

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We have cloned and sequenced the *nrd* (nucleotide reductase) locus of *Bacillus subtilis*. The locus seems to be organized in an operon comprising four ORFs. The first three encode polypeptides highly similar to the product of the coding sequences characterizing the *nrdEF* operons of *Enterobacteriaceae*. The sequencing of the conditional lethal mutation *ts-A13*, localized in the *nrdE* cistron, and the lethality of insertional mutations targeted in the internal region of *nrdE* and *nrdF*, demonstrated the essential role of this locus. The fourth ORF, *ymaB*, part of the putative operon, which is not similar to any known protein, is also essential. The regulation of expression of the operon, monitored by *lacZ* transcriptional fusions, is similar to the regulation of the functionally relevant *nrdAB* operon of *Escherichia coli*. The operon was induced by thymidine starvation and its expression was directly or indirectly affected by RecA function. Genetic and functional analysis strongly indicates that in *B. subtilis* the class I ribonucleotide reductase encoded by this *nrd* operon is evolutionarily distant from the homologous class I enzyme of *Enterobacteria*.

Keywords: *Bacillus subtilis*, genome sequencing, ribonucleotide reductase, *nrdEF* operon, *ts-A13* mutation

INTRODUCTION

Ribonucleotide reductases (RRs, ribonucleoside diphosphate reductases) are enzymes essential for the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides. In addition they exert an important role in balancing the supply of the four dNTPs and consequently in the control of DNA synthesis. In prokaryotes the presence of three classes of enzymes has been documented (Reichard, 1993). Class I enzymes, the best known from structural studies (Nordlund *et al.*, 1990; Nordlund & Eklund, 1993; Uhlin & Eklund, 1994), are tetrameric, aerobic and use, for the generation of the

organic radical necessary in the initial step of the enzymic reaction, an iron-containing protein, represented by the R₂ homodimer. The R₁ homodimer contains the allosteric binding sites and the catalytic site. Class II enzymes, present in different aerobic and anaerobic prokaryotes, are monomeric and use adenosyl cobalamin as radical generator (Booker & Stubbe, 1993) taking over the function of the R₂ protein of the class I enzymes. The amino acid sequence has no similarity with R₁ with the exception of nine amino acids at the C terminus. The class III enzyme was discovered in *Escherichia coli* growing anaerobically. It is a homodimer with an iron-sulfur centre and an oxygen-sensitive protein radical probably represented by a Gly radical close to the C terminus (Sun *et al.*, 1993). A similar function is encoded by bacteriophage T4. Comparison of the amino acid sequences of class I enzymes of different species suggested the existence of three different groups, namely eukaryotic RR, herpesvirus-type enzymes, and *E. coli* and bacteriophage T4 *nrdAB* gene products (Nordlund & Eklund, 1993), with a very low level of similarity between them (at the boundary of significance, between 20 and 30% identity). Recently, the genes encoding a

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Abbreviations: *nrd*, Nrd, nucleotide reductase; RR, ribonucleoside diphosphate reductase; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

The GenBank accession number for the sequence reported in this paper is Z68500.

fourth group of class I enzymes were identified in *Salmonella typhimurium* and *E. coli* and named *nrdEF* (Jordan *et al.*, 1994a). This new operon, when carried on a multicopy plasmid, can complement *nrdAB* conditional lethal mutants in standard growth conditions, suggesting that it encodes an aerobic function, usually expressed at low level. The deduced products of *nrdEF* showed only limited homology with the other groups of class I enzymes (between 19 and 27% identity) but many of the catalytically important amino acid residues were conserved. The products of the two genes, *nrdE* and *nrdF*, are homodimeric proteins: the R1_ER2_F enzyme is able to catalyse the reduction of CDP to dCDP in the presence of dithiothreitol or reduced glutaredoxin as an electron donor, instead of thioredoxin, and its allosteric regulation is different from that exhibited by the enzyme encoded by the *nrdAB* operon (Jordan *et al.*, 1994b).

In *E. coli* the expression of the *nrdAB* operon is cell-cycle-regulated (Filpula & Fuchs, 1977, 1978; Sun & Fuchs, 1992) with a *cis*-acting positive A-T-rich element (Sun *et al.*, 1994) as regulator. Two *trans*-acting positive regulators are DnaA and Fis (Augustin *et al.*, 1994), but these proteins are not required for the coupling of *nrdAB* operon expression to DNA synthesis during the cell cycle. The activity of RR and the transcription of the operon are both increased when DNA replication is inhibited (Hanke & Fuchs, 1983) and after DNA damage (Gibert *et al.*, 1990), but this increase seems to be only indirectly related to DNA damage.

In *Bacillus subtilis* the isolation and characterization of a temperature-sensitive mutant (*ts-A13*) led to the identification of the *nrd* locus encoding a RR, functionally similar to the aerobic class I RR of *E. coli* (Bazill & Karamata, 1972). In a marker rescue screening, a clone positive for the *ts-A13* allele (previously named *dnaA-13*) was isolated from a plasmid bank of *B. subtilis* genomic DNA (Perego *et al.*, 1987). The *nrd* (*dnaA*) locus was mapped at about 167° on the chromosome, between the *gluRA* operon and the *pks* locus (Fisher *et al.*, 1984; Gianni & Galizzi, 1986; Scotti *et al.*, 1993). In this paper we describe the molecular cloning and sequencing of the entire *B. subtilis* *nrd* locus, the localization and nature of the *ts-A13* mutation, gene inactivation experiments and preliminary results on the regulation of expression of the locus.

METHODS

Bacterial strains, plasmids and media. *B. subtilis* strains used for gene inactivation and transcriptional fusion are listed in Table 1. *E. coli* DP50 (*supF* [*F*⁻ *tonA53 glnV-44 tyrT-58 dapD-8 lacY-1 supE-44 supF-58 Δ(gal-uvrB)47 gyrA-29 thyA-57 hsdS-3*]) and LE392 (*supE-44 supF-58 hsdR-514 galK-2 galT-22 metB-1 trpR-55 lacY-1*) were used as hosts for λ Charon 4A recombinant derivatives. Cultures were grown in TY medium (tryptone-yeast extract medium supplemented with 100 μ g diaminopimelic acid ml⁻¹ and 50 μ g thymidine ml⁻¹ in the case of strain DP50) or LB medium.

Antibiotics were added at the following concentrations: ampicillin (Ap), 100 μ g ml⁻¹; chloramphenicol (Cm), 20 μ g ml⁻¹ for *E. coli* and 2.5 or 5 μ g ml⁻¹ for *B. subtilis*; kanamycin (Km), 2 μ g ml⁻¹. *E. coli* DH5 α [*supE44 lacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17*

recA1 endA1 gyrA96 thi-1 relA1] was used for plasmid transformation and propagation. The genomic *B. subtilis* λ Charon 4A bank was made with DNA from strain 168 (*trpC-2*) (Ferrari *et al.*, 1983). The insert from the λ CH4G621 clone was sub-cloned in the pJM103 integrative vector (Perego, 1993); the integrative vectors pJM783 and pJM115 were used to generate transcriptional fusions to *lacZ* (Perego, 1993).

Two transcriptional fusions were constructed, one placing the reporter gene, *lacZ*, downstream of the whole operon to evaluate the expression of the operon. The second fusion was constructed to test the presence of a putative promoter between *nrdF* and *ymaB* (Fig. 1). This fusion was inserted into the *amyE* locus.

A 538 bp fragment corresponding to the 3' end of *ymaB*, with artificial *EcoRI* and *BamHI* sites, was produced by PCR using the primers 5' GGAATTCAGTGAAGATAGCAGAGTAGCA 3' and 5' GCCCTAGGTTATTCAAGAATATCAACAACA 3' [the residues corresponding respectively to nt 4533–4554 and 5049–5070 of Z68500 (GenBank accession no.) are underlined]. Following purification and restriction, the fragment was cloned into pJM783 (Perego, 1993).

The plasmid obtained, pM783/538 EB, was used to transform competent cells of strains PB168 or PB1831, selecting for Cm^R. The integration by single crossover in the region of homology, provided by the insert, placed the *lacZ* reporter gene downstream of the whole operon. The construction was verified by Southern hybridization.

A 487 bp fragment with artificial *EcoRI* and *BamHI* ends, corresponding to the intercistronic region between *nrdF* and *ymaB*, was synthesized by PCR using the primers 5' GGAA-TTCCTACAGCGGTTTCTATTAT 3' and 5' CGGGATCC-TTATATCTGCTCTTTTTCAT 3' corresponding respectively to nt 3914–3932 and 4400–4381, and cloned in the vector pJM115. The derived plasmid, named pJM115/487 EB was linearized and used to transform competent cells of strain PB168 selecting for Km^R. Double crossover events caused by the insertion of the *nrdF-lacZ* fusion at the *amyE* locus gave an Amy⁻ phenotype.

The other plasmids used for sequencing and insertional gene inactivation of the three main coding sequences of the *nrd* operon were constructed by cloning suitable restriction fragments in the integrative vector pJM103 and are partially listed in Table 2.

Transformation. *E. coli* transformation was performed following standard protocols (Hanahan, 1985). *B. subtilis* competent cells (Hoch *et al.*, 1967) were transformed using 0.5–1 μ g plasmid DNA or 0.02–0.1 μ g chromosomal DNA (Albertini & Galizzi, 1985).

Sequencing. Sequencing was performed on plasmid subclones in pJM103 (Table 2) obtained from the *EcoRI-SalI* fragment of λ CH4G621 as indicated in Fig. 1. The overlapping *HindIII* fragment was amplified by reverse PCR, recovering a fragment of 1.87 kb (dashed in Fig. 1) from chromosomal DNA digested with *HindIII*, ligated and cut at the unique *SacII* site at nt 1782. Amplification was obtained with primers NRDA3, 5' CGCA-CTCATGAAGGAAGGAAAACGA 3', and NRDA4, 5' GAGCAATCGATATCTCCATGCAGCT 3'. The plasmid subclones were sequenced with Sequenase (USB) or T7 (Pharmacia) sequencing kits. The reverse PCR product was sequenced with the Circumvent kit (Biolabs). In all cases the sequence was determined on both strands by primer walking using custom oligonucleotides synthesized with a Cyclon Plus Synthesizer (Milligene Bioscience).

Table 1. *B. subtilis* strains used in this study

Strain	Genotype*	Reference or source†
PB168	<i>trpC-2</i>	J. Lederberg E3113 (D. Karamata) JH 949 (J. Hoch) JH642 (J. Hoch) BGSC 1A746
PB566	<i>trpC-2 thyA thyB</i>	
PB1679	<i>ilvA-1 metB-5 ts-A13</i>	
PB1783	<i>thyA thyB glnA-100</i>	
PB1831	<i>trpC-2 phe-1</i>	
PB1843	<i>trpC-2 recA-260 metB-5 SPβ(-) xin-1 erm</i>	
PB1848	<i>trpC-2 phe-1 nrd::pJM783/538 EB nrd-lacZ cat</i>	
PB1849	<i>trpC-2 nrd::pJM783/538 EB nrd-lacZ cat</i>	
PB1850	<i>trpC-2 thyA thyB nrd::pJM783/538 EB nrd-lacZ cat</i>	
PB1851	<i>trpC-2 phe-1 ΔcomP-A nrd::pJM783/538 EB nrd-lacZ kan cat</i>	
PB1852	<i>trpC-2 recA-260 nrd::pJM783/538 EB nrd-lacZ erm cat</i>	
PB1854	<i>trpC-2 thyA thyB ΔcomP-A nrd::pJM783/538 EB nrd-lacZ kan cat</i>	
PB1855	<i>trpC-2 thyA thyB recA-260 nrd::pJM783/538 EB nrd-lacZ erm cat</i>	
PB1874	<i>trpC-2 phe-1 amyE::pJM115/487 EB nrd-lacZ kan</i>	
PB5091	<i>trpC-2 ΔcomP-A kan</i>	QB4714 (T. Msadek) JH646 (J. Hoch)
PB5137	<i>trpC-2 phe-1 spo0A-12</i>	

* *cat*, *kan* and *erm* are the determinants for resistance to chloramphenicol, kanamycin and erythromycin, respectively.

† BGSC, Bacillus Genetic Stock Center, Ohio, USA; J. Hoch, La Jolla, USA; D. Karamata, Lausanne, Switzerland; J. Lederberg, New York, USA; T. Msadek, Paris, France.

Fig. 1. Physical map and gene organization of the *nrd* locus of *B. subtilis*. (a) Clone λCh4G621. (b) Map of the sequenced region. The hatched bar represents the sequence obtained by reverse PCR; arrows indicate the extent of the coding sequences; hairpins indicate putative terminator or attenuator structures; boxes indicate the localization of the fragments cloned in relevant plasmids. H, *Hind*III; E, *Eco*RI; S, *Sal*I; P, *Pst*I; S2, *Sac*II. Δ indicates the position of the *ts-A13* mutation.

Table 2. Plasmids used in this study

Plasmid	Description (source or reference)
Vectors	
pJH101	Ap Tc Cm; integrative vector (Ferrari <i>et al.</i> , 1983)
pJM103	Ap Cm; integrative vector (Perego, 1993)
pJM783	Ap Cm; vector used to construct a <i>lacZ</i> transcriptional fusion (Perego, 1993)
pJM115	Ap Km; vector used to construct <i>lacZ</i> transcriptional fusion and recombine into the chromosome at <i>amyE</i> , derivative of pDH32 (Shimotsu & Henner, 1986)
Other plasmids	
pMP25.8	Ap Tc Cm; pJH101 derivative (Perego <i>et al.</i> , 1987)
pJM780	Ap Cm; 0.7 kb <i>EcoRI</i> – <i>SaI</i> fragment in pJM103
pJM3000	Ap Cm; 3 kb <i>SaI</i> fragment in pJM103
pJM606	Ap Cm; 0.6 kb <i>HindIII</i> fragment subcloned from pJM3000 in pJM103
pJM244	Ap Cm; 0.2 kb <i>HindIII</i> fragment subcloned from pJM3000 in pJM103
pJM231	Ap Cm; 0.2 kb <i>HindIII</i> – <i>RsaI</i> fragment subcloned from pJM3000 in pJM103
pJM783/538 EB	Ap Cm; 0.5 kb <i>EcoRI</i> – <i>BamHI</i> linked PCR fragment in pJM783
pJM115/487 EB	Ap Cm; 0.4 kb <i>EcoRI</i> – <i>BamHI</i> linked PCR fragment in pJM115

Sequencing of the *ts-A13* mutation. The products obtained from two independent experiments of PCR amplification of chromosomal DNA of mutant strain PB1679 using the primers at nt 1872 (5' CTTGGTGGCGGGGTTTCT 3') and nt 2685 (5' AGCACCGAGGCCGATTGA 3') were digested with *HindIII* and cloned in pJM103 to be sequenced on both strands. We considered as mutations only the base substitutions verified on clones obtained from both the independent PCR reactions.

β -Galactosidase assay. β -Galactosidase activity in strains harbouring *lacZ* transcriptional fusions was measured as described previously (Albertini & Galizzi, 1990).

DNA manipulations. Plasmid and phage DNA preparations and Southern hybridization were performed according to standard protocols (Sambrook *et al.*, 1989). *B. subtilis* chromosomal DNA was prepared by phenol extraction (Albertini & Galizzi, 1985).

RESULTS AND DISCUSSION

Cloning and sequencing of the *nrd* locus

Plasmid clone pMP25.8 (Perego *et al.*, 1987) was able to marker rescue the temperature-sensitive mutant *ts-A13* in transformation experiments and direct selection to *ts*⁺. None of the transformants was Cm^R. This observation indicated that integration by single crossover is probably lethal and that the insert of the plasmid was internal to the *nrd* locus and not inclusive of the 5' or 3' boundaries. For this reason we started screening a λ Charon 4A bank (Ferrari *et al.*, 1981) by plaque hybridization using labelled pMP25.8 as probe and identified one positive clone, λ G621, with an insert of about 13 kb (Fig. 1). The restriction map of the clone was similar to that of clone λ 4A-2T-4 isolated from the same bank and bearing the sequence corresponding to the *ts-A13* and *glnRA* loci (Fisher *et al.*, 1984). DNA from phage λ G621 did in fact

transform *glnA-100* to prototrophy, supporting the idea that the two clones are identical.

We subcloned the 780 bp *EcoRI*–*SaI* fragment of pJM780 and the 3283 bp *SaI* fragment of pJM3000 in pJM103 (Perego, 1993) and sequenced by primer walking. Analysis of the sequence revealed the existence at the 5' end of an incomplete ORF showing similarity to the major subunit of class I RRs. To extend the cloning in the 5' direction, we tried, unsuccessfully, different strategies, i.e. chromosome walking with various integrative plasmids and screening of different *B. subtilis* λ or plasmid libraries. The sequence was obtained by reverse PCR and direct sequencing of the PCR product (see Methods).

Organization of the *nrd* locus

Analysis of the sequence comprising the *nrd* locus and the surrounding region, performed using the BLAST (Altschul *et al.*, 1990) and FASTA (Pearson, 1990) collections of programs, revealed the following features. The first 865 bp are characterized by short, less than 279 bp ORFs on both strands, whose deduced products did not show any similarity to known proteins. Nevertheless, we found the consensus for a putative σ A promoter at nt 51–80 and a hairpin-loop structure (ΔG –114 kJ mol^{–1}) at nt 745–776, suggesting that the sequence corresponds to a transcription unit without coding capacities.

A second consensus for a putative σ A promoter could be recognized between nt 865 and 891, followed at nt 921–926 by a putative ribosome binding site just upstream of the GTG start codon of an ORF, named *ymaA* (Fig. 1). The predicted polypeptide of 130 aa (expected *M_r* 14600) showed significant similarity to the deduced product of

short ORFs of unknown function just upstream of the *nrdEF* operons of *S. typhimurium* (ORFB) and *E. coli* (ORFC) (Jordan *et al.*, 1996). A similar polypeptide was also predicted in *Lactococcus lactis* (GenBank accession no. X92690) and in *Mycoplasma genitalium* (predicted coding region MG230, Fraser *et al.*, 1995). In all cases the ORFs seem to be part of an *nrdEF* operon.

A ribosome binding site consensus at nt 1276–1283 precedes an ORF encoding a polypeptide of 700 aa, with a predicted M_r of 80700, showing high similarity (identity score 44–53%) to the second group of enterobacterial class I RRs described by Jordan *et al.* (1994a, b). The same type of RR was isolated from *Mycobacterium tuberculosis* (Yang *et al.*, 1994) and deduced to be present in *Myc. genitalium* (Fraser *et al.*, 1995). The high similarity is evident in the multiple alignment shown in Fig. 2, and extends beyond the conserved three cysteines of the essential thiol, characterizing all the large RR subunits of class I. As in the case of the NrdE subunits of *Enterobacteriaceae*, the similarity with the prokaryotic NrdA subunit is low (22% identity), even lower than the similarity observed with the eukaryotic RR major subunits (25% identity).

A ribosome binding site consensus at nt 3397–3401 precedes a sequence (nt 3411–4400) potentially encoding a polypeptide of 329 aa, with an expected M_r of 38400, with high similarity to the second group of class I minor subunits of RR, namely NrdF, found in *Enterobacteriaceae* (Jordan *et al.*, 1994a, b) and *Myc. genitalium* (Fraser *et al.*, 1995). Fig. 2 shows the alignment between the NrdF polypeptides sharing identity scores between 39 and 54%. In this case as well, the similarity extends beyond the conservation of residues involved in defined functions such as the major subunit binding surface, the environment of the tyrosyl residue, the hydrogen bonding network, the iron ligands and the four α -helix domains surrounding them. The similarity with other class I minor subunits is lower, ranging from 23% identity with the eukaryotic ones and 21% with NrdB of *E. coli* or bacteriophage T4.

No terminator site or recognizable promoter was identified between *ymaA* and *nrdE*, or between *nrdE* and *nrdF*. In addition, the general organization of the coding sequences is very similar to that of the characterized *nrdEF* operon of *S. typhimurium* and *E. coli* (Jordan *et al.*, 1996). We thus propose that the three ORFs belong to the same operon.

It should be pointed out that the *B. subtilis nrd* operon, even though highly similar to the enterobacterial *nrdEF* operon, lacks a gene encoding a polypeptide similar to glutaredoxin, the proposed electron donor for the NrdEF enzyme (Jordan *et al.* 1996). Such an ORF is not present even in the proximity of the *nrd* locus.

The stop codon of the putative NrdF coding region is part of the loop of a stem-loop structure with a stability of -114 kJ mol^{-1} , followed by a putative ribosome binding sequence (nt 4432–4439) which precedes an ATG codon. The following ORF (nt 4450–5070), named *ymaB*, could

encode a polypeptide of 206 aa, with a predicted M_r of 23400. It should be noted that a partial consensus to a σA promoter, which could drive the transcription of *ymaB*, is present between nt 4345 and 4372.

The hypothetical product YmaB is devoid of similarity to known proteins or to hypothetical products present in data banks. The ORF is followed by a hairpin-loop structure (Fig. 1) that could act as a strong ($\Delta G -169.9 \text{ kJ mol}^{-1}$) terminator for the *nrd* operon and for the transcription unit on the complementary strand (ORF from nt 5559 to 5134), that corresponds to the 3' end of the *culC* gene described by Kuroda *et al.* (1993).

Inactivation of the three main coding sequences

The three subclones pJM606, pJM244 and pJM231 (described in Table 2) were used as insertional vectors to specifically interrupt, by insertion of the plasmid, the three major ORFs, i.e. *nrdE*, *nrdF* and *ymaB*, respectively. In all instances the restriction fragments used to target the insertion of the plasmid in the chromosome forced the recombination event in an internal region of the gene as indicated in Fig. 1.

We used these plasmids to transform competent cells of *B. subtilis* to Cm^R and we never obtained transformants. This result is similar to that obtained with plasmid pMP25.8. Since we had no difficulty in obtaining transformants by a single crossover event using plasmid pJM3000, bearing the C terminus of *nrdE* and the entire coding regions of *nrdF* and *ymaB*, we deduced that the interruption of the operon was probably lethal and that at least the further downstream ORF *ymaB* encodes an essential function.

Mapping and sequencing of the *ts-A13* mutation

Bazill & Karamata (1972) analysed the conditional lethal mutant, *ts-A13*, impaired in ribonucleotide reductase activity. It is the only conditional lethal mutant reported for this function in *B. subtilis*, suggesting that the mutation impairs the functionally relevant class I enzyme of this organism.

Perego *et al.* (1987) isolated pMP25.8, an integrative plasmid able to marker rescue to ts^+ the temperature-sensitive mutant *ts-A13*. To define more precisely the region of the mutation, we subcloned a number of DNA fragments and tested them in marker rescue experiments.

Among the subclones, plasmid pJM606, unable to transform cells to Cm^R , was able to give ts^+ transformants when used to transform strain PB1679. The same *HindIII* fragment was obtained by PCR amplification from chromosomal DNA of the mutant strain PB1679 and was sequenced. We found three mutations: two in close proximity (GC $\rightarrow$ AT transition at nt 2292; AT $\rightarrow$ GC transition at nt 2295) were silent substitutions in the third position of the codons for Lys333 and Glu334 of NrdE and the third mutation was a CG $\rightarrow$ TA transition, changing His255 of NrdE to Tyr. This histidine residue is conserved in all class I major subunits of RR so far examined, and corresponds to the last residue (His308) of

(a)

NrdEB.s.	M-----SQNQVPKWIQLnei-----	68
NrdES.t.	Mqetm-----DYHANAMHLYKASHIQKQQAIDAFATVRPHVTFAQHEHLCGLREGAIDAV	67
NrdEM.g.	MtskekiptfntEEDVESYISFhaqaklydd-----	74
NrdEM.t.	ptviaepvasgahasysggpgedyHANAMHLYADAKIKIKREAAHQYQLQVNNQNVFSHIODEFTLYITRENTEREV	85
NrdEB.s.	LSLSFEDIKEFKTAYAKKFFPSGSAKFNDAKFNDRPKLRRFPSTVFNTANTTEKKEYVNLINQNYQCT	153
NrdES.t.	IARFIRAFVLRFEHHAHSGFPFQTFGAWKFTSTTKHEDGRRLDEHEFTVTVAITLAQDETILTQLTDEMLSGAFQTA	152
NrdEM.g.	INMGFEQFEETHTKAYSYRFFYANMGAPKFNANAKTFTGKYTLENNEIFAVNNVIMLANNNFKLKLKQITLNFQTA	159
NrdEM.t.	IDQSRNFVKTLDRAYAKKFFPTLGAFTYTSITKTFDGRRLRRFELRVVVAITLAANTALDELLVDEIDGTFQTA	170
NrdEB.s.	ITPLHAHARRGELVSFILFVNTHNLISPAIDISIQRLRLGAVNNLSNLPKKEALIDVEATKSVVWNNLLNARRAL	238
NrdES.t.	PTFLNCKKQORGELVSFILFIEMHEDIGFANSALQIRRGGVALLSHLFAAPAPIRIENSTQVTPMREMLGANSAAH	237
NrdEM.g.	PTFLNARRKRGELVSFILFIEIMHEDIGFANSALQIRRGGVALLSHLFAAPAPIRIENSTQVTPMREMLGANSAAH	244
NrdEM.t.	PTFLNSKRRGELVSFILFVEIMHEDIGFANSALQIRRGGVALLSHLFAAPAPIRIENSTQVTPMREMLGANSAAH	255
NrdEB.s.	QMGQRGGGAALNIFHFDINDLETKRISADELVFVETISIGVILIKFNEIAREIKARYTYHTIYREYQHMDEEDMMEM	323
NrdES.t.	QLGARQGAGAVLHAHHPIMLRLFTKRENAIFETIKTNLGGVILITERRARENAQVANSSEIQRKAPFGFANARR	322
NrdEM.g.	QLGARQGAGAVLLECHPIAMQGLTKRENAIFETIKTNLGGVILITERRARENAQVANSSEIQRKAPFGFANARR	329
NrdEM.t.	QLGARQGAGAVLHAHPIMLRLFTKRENAIFETIKTNLGGVILITERRARENAQVANSSEIQRKAPFGFANARR	340
NrdEB.s.	DRFDINPVAKKKINPKILERLAMRDESGFTINPMNVNVAHHNHSYKFTIDSNVDAQVVSSTDYDEEDEELIYS	408
NrdES.t.	DELIAEPVVKTYINARDPQITAEQQLSTGCTINFEITVNHAN--PIAGNNMHLSSEIAVVSASRDDNLDYTHGHIF	405
NrdEM.g.	YELDENQIKKTESARKFFETIAEHFESECTIDFEITVNHAN--RNAHKNIVMENLSHIVAPLPSEYSDLTFKKQGSIF	412
NrdEM.t.	YEMDIAIRIKTKIKAREFEQTLAEQFEGGCTIMFEITVNHAN--PIDGNITHRLSELGVTPSTENEDLSYAKGKIS	423
NrdEB.s.	CNLGSLNLLNVMEHKS-IEKTVRLTDSITHSETIDRNADVRRRAKAMASISGATHHGGYLAQNGIAREEPARQATTF	492
NrdES.t.	CNLGSLNIAHVMISPD-IGRTVETAIRGTHAMSDMHSRVSPIAANHAASIAIGTCQNLHGYLAREGANGSPALPTMLIF	489
NrdEM.g.	CNLGSLNIARAMESSGSeLAELICLAIESILLRSISSIETAPSIKKNSENHALGLCANLHGLATNAYYDKEAVDPTIIF	497
NrdEM.t.	CNLGSLNIAKTMISPD-FACTIEVIRANTARHCHTHKSVSEIEQNNDSHAIGLQCNLHGYLARERIFSGDEGILEFTIYF	507
NrdEB.s.	MMVNFYSIQRSADIAKKEKETDQEGGTYATETET---DRTVSTDSPRYEKANLDE--AHEFTTIDDKTKAFVAEHMYH	572
NrdES.t.	YTIIVHAVHTCMRLARERKKTAGFAQRYAS---HYTTLQDDQPKTARALHARSITHTIRAMMLKTRDDMYRYGIYD	571
NrdEM.g.	YTVVAHAFSANKLLELCK-KKDPENKFPADSTIKKTYMASDEWTCTETEQALDKYAKKIQQNMKQLVASIQDKGLAN	581
NrdEM.t.	YTVVHALRASNRALERTHKKGFERRKYS---HFDNVTDQTEPKTCVRQLADARIRLQDWRALKESVQAHSTIYH	589
NrdEB.s.	SYRLKANTGSISYQSSIASVMEIHERTEENTYNSKTYPIPLGLASINWFFKKE--ATTHMFVVMISTIQCHIDQISFT	655
NrdES.t.	QNLCAMPPTGGSITYNHASSHPVIAKTEPTEENTFVYAPFMTNENLDYQ-DAVETPERIITYEATARVLOQSGIT	655
NrdEM.g.	SHLMHAATGGSISYSSCPSPKCVVSPTEVEEELIIVAVKLDHINYQTEGAEIYGFPIIDVNAQAQVYVAIAHIT	666
NrdEM.t.	QNLCAMPPTGGSISYNHSASSHIVSKTEVEEELIIVYAPYMTNINLEYQ-DAVEIGYESIITYAATQIVEGGLSLT	673
NrdEB.s.	LFLKTMITRLINRIDYAHHRGIKTIVAAKTKT-----GQ---DSLSLVV	700
NrdES.t.	LEFFETANTRLHKAQYAWRKGIKELYILPALALEG-----E---EGVVSAL	705
NrdEM.g.	LEFMTIRANTRLINRAYIAFKKGCSSITVVPQDVLDsedhtiki--KDREVSI	721
NrdEM.t.	LEFFKUTATRLVNKAQYAWRKGIKELYILPALALEG-----E---EGVVSME	723
NrdFB.s.	WtKiYd-----	63
NrdFS.t.	M-Klsr-----	64
NrdFM.g.	MaannkkyflesfplgyvknfngnRNVWLVLDDEKDVLRIVQFWLPEKIPVNDLPSWAKLSKDWGLTITITELT	85
NrdFE.c.		0
NrdFB.s.	LLDTEGNTMPIVAEIVDGHARKANLNFMAHENAIAKESNTNFMADAPETITNEVFVWQKYQKRAAMGLkaikqd	148
NrdFS.t.	LLDTICNIAAPSILMAITPHEEALSNISFTE-AVIAESSTSLQIREVIAAYAMSEEPAPAKAIIIAHVSDEPL	148
NrdFM.g.	LLDTICATIDICQIDHALTDIEQVIYANFAFW-GVIAESYGTISLCTEQVIAAHEWVNNESLQKRAALPYITGNDPL	169
NrdFE.c.		0
NrdFB.s.	deislFAMVASVYTESLFPSTFYFLFYGGGMMSGEINNTLRDPAITHGVVLLAEIYNQTEEKAKARREFAIDIN	233
NrdFS.t.	-----KKLASVIESLFPSTFYLMMFSSGGITNTAPLRUIIRDAVIGYIYKYIALQNSAIEBELFLPALILM	227
NrdFM.g.	-----SKVAALMPGELLYGHEFLFSSKKIPHTSDIRLLEFHVILYVSYKYARKLESLPLAKKPMHAFPEFLMY	248
NrdFE.c.	-----ISLGOEELISAEFLGL	18
NrdFB.s.	QLYNLELYEDLDQVGLSHAKKIRNAHRAHMLGFTPY--DEEDNHLVINGLTKRTHHFE--MKGHCRRKTVEL	315
NrdFS.t.	ELYNLEIRTEALAEETGWNVAKALCNHAKKALMHGYBALPPMADAHAIHAAASPNALEHDDFGSGSSVMAKTVEF	312
NrdFM.g.	RLIELEKDYKELLEGFIVDLAIKSVTHAGKFLQNGYLSPTAEETRNEIHAQLSARLEHDDFGSGSSVMAVSEET	333
NrdFE.c.	ELYNLELQWDELAEETPWADLAKLQTHAKKALMHGYPEPLPAPMAEVNIAHAAASPNALEHDDFGSGSSVMAKAVET	103
NrdFB.s.	KDDEFFYedekeqi	329
NrdFS.t.	EDEDWNS-----	319
NrdFM.g.	NDETWNS-----	340
NrdFE.c.	EDEDWNS-----	110

Fig. 2. For legend see facing page.

Fig. 3. Phylogenetic analysis of the class I major subunits (a) and minor subunits (b) of RR graphically displayed using the NJPLOT software of M. Gouy (University of Lyon, France). The scale bars indicate the distances as calculated from the multiple alignment (Thompson *et al.*, 1994). Species abbreviations and protein or DNA sequence accession numbers used for (a) are: NrdEB.s., *B. subtilis* (Z68500); NrdES.t., *S. typhimurium* (Q08698); NrdEM.t., *Myco. tuberculosis* (L34407); NrdEM.g., *Myc. genitalium* (U39701); NrdAE.c., *E. coli* (P00452); NrdAH.i., *H. influenzae* (P43754); NrdAS.t., *S. typhimurium* (P37426); NrdAT4, bacteriophage T4 (P32282); Rir1S.p., *Schizosaccharomyces pombe* (Z67998); Rir1H.s., *Homo sapiens* (P23921); Rir1C.e., *Caenorhabditis elegans* (Q036304); Rir1V.v., *Vaccinia virus* (P20503); Rir1P.f., *Plasmodium falciparum* (A49412); Rir1S.c., *Saccharomyces cerevisiae* (P21524). For (b): NrdFB.s., *B. subtilis* (Z68500); NrdFS.t., *S. typhimurium* (P17424); NrdFE.c., *E. coli* (P37146); NrdFM.g., *Myc. genitalium* (P47471); NrdBE.c., *E. coli* (P00453); NrdBS.t., *S. typhimurium* (P37427); NrdBH.i., *H. influenzae* (P43755); NrdBT4, bacteriophage T4 (P11156); Rir2P.f., *P. falciparum* (B49412); Rir2H.s., *H. sapiens* (P31359); Rir2He.s., *Herpes simplex virus* (P28847); Rir2S.s., *Spizula solidissima* (P07201); Rir2N.t., *Nicotiana tabacum* (X92443); Rir2C.e., *C. elegans* (P42170); Rir2S.p., *Schiz. pombe* (P36603); Rir2S.c., *Sacch. cerevisiae* (P09938); Rir2D.d., *Dictyostelium discoideum* (P42521).

the β C sheet of the α/β barrel domain described in the three-dimensional structure of the NrdA subunit (Uhlin & Eklund, 1994). We infer that this mutation is the one responsible for the temperature-sensitive phenotype of the *ts-A13* mutant allele.

The identification of three mutations, two neutral and one that is presumably responsible for the conditional lethal phenotype, in a short stretch of DNA sequence can be explained by the fact that the mutant *ts-A13* was isolated

after NTG treatment, a mutagen which acts on the DNA replicative fork and is known for inducing multiple mutations (Karamata & Gross, 1970).

The localization in *nrdE* of the mutation that impairs the activity of the class I RR (Bazill & Karamata, 1972) and the evidence that the *nrdEF* operon cannot be inactivated led us to infer that this operon exerts a role essential for the biosynthesis of deoxyribonucleotides. It seems safe to conclude that the NrdER₁/NrdFR₂ enzyme plays the

Fig. 2. Multiple alignments (a) of NrdE polypeptides, major subunits of RR, and (b) of NrdF polypeptides, minor subunits of RR, found in *B. subtilis* (B.s.), *E. coli* (E.c.), *S. typhimurium* (S.t.), *Myc. genitalium* (M.g.) and *Myco. tuberculosis* (M.t.). GenBank accession numbers are given in the legend to Fig. 3. (a) In the NrdE alignment, ● indicates the Cys residues proposed for the 3' hydrogen abstraction of the substrate and ▼ indicates the two Cys residues of the active site. (b) In the NrdF alignment, the functionally relevant conserved residues are indicated by the following symbols: ▼, hydrogen bonding network sites; ●, residues of the tyrosyl radical environment; ○, residues of the major subunit binding surface; *, iron ligand residues.

Table 3. Expression of the *nrd* operon

The indicated values represent the mean of at least two experiments and were the highest obtained from a series of samples assayed at various times during growth. ND, not determined.

Strain	β -Galactosidase activity [U (mg protein) ⁻¹]* (<i>nrd::pJM783/538 EB nrd-lacZ</i> fusion)			
	SM medium	SM medium (-)	TM medium	TM medium (-)
PB1848 wild-type	13	14	8	9
PB1849 wild-type	12	ND	14.2	ND
PB1850 Thy ⁻	28.5	35	23.1	50.6
PB1851 Com ⁻	11.5	ND	8.5	ND
PB1852 Rec ⁻	10	ND	8	ND
PB1854 Com ⁻ Thy ⁻	22	33.5	28.5	52
PB1855 Rec ⁻ Thy ⁻	21	28.2	21.5	16.5

* SM, Schaeffer Medium; TM, competence minimal medium (Anagnostopoulos & Spizizen, 1961). Both media were supplemented with 50 µg thymidine ml⁻¹. (-) indicates thymidine deprivation.

same role in *B. subtilis* that the NrdAR₁/NrdBR₂ enzyme plays in *Enterobacteriaceae*. In *E. coli* and in *S. typhimurium*, the *nrdEF* operon, even if expressed, is not essential, and in standard conditions is unable to replace the absence of NrdAR₁/NrdBR₂ activity (Jordan *et al.*, 1996). Although at present we cannot rule out the hypothesis that an *nrdAB*-type operon is present in the *B. subtilis* genome, it appears that *B. subtilis* made a functional choice concerning the RR enzyme different from that made by the *Enterobacteriaceae*. The same choice was made by *Myc. genitalium*: in the whole genome only one locus is devoted to deoxyribonucleotide biosynthesis and it appears to be organized in an operon with three ORFs, the *nrdF* homologue, the homologue of *B. subtilis ymaA* and the *nrdE* homologue. With the exception of the relative order of genes, the genetic tools present in *Myc. genitalium* are the same as found in *B. subtilis*. *Haemophilus influenzae*, the other bacterium for which the entire genome has been sequenced (Fleischmann *et al.*, 1995), made the opposite choice: only an *nrdAB* operon is present.

Prokaryotes evolved different strategies for deoxyribonucleotide production (Reichard, 1993), namely class I and class II aerobic and class III anaerobic enzymes. The three classes are not related at the level of primary structure. In addition, if we examine the variety of aerobic Fe(III)-tyrosyl radical-based class I enzymes, a category comprising the eukaryotic enzymes, we observe a wide diversification at the level of primary structure. Fig. 3 represents the phylogenetic analysis of RR major and minor subunits: the NrdER₁/NrdFR₂ RR diverged from NrdAR₁/NrdBR₂ enzymes at least as much as both diverged from the eukaryotic enzymes. In the case of the major subunits, the NrdA-type polypeptides are more similar to the eukaryotic counterpart than to the NrdE prokaryotic polypeptide.

New questions arise from these observations. Why did

Enterobacteriaceae and *H. influenzae* make a functional choice (in the case of *Enterobacteriaceae*, maintaining both the genetic equipment and the production at low levels of the NrdER₁/NrdFR₂ enzyme) different from *B. subtilis* and *Myc. genitalium*? Is it possible that an ancestor of *Enterobacteriaceae* acquired the *nrdAB* genes from a bacteriophage, such as T4, which has an RR highly similar to the NrdAR₁/NrdBR₂ enzyme, and the newly acquired enzyme has been raised to a higher functional role in the course of evolution? Is the choice of the *Enterobacteriaceae* in some way related to the presence of the anaerobic *nrdD*-encoded class III enzyme, proposed as the closest relative of the common ancestor of all RRs (Reichard, 1993)? In more general terms, why do we find class II enzymes in Eubacteria (e.g. *Lactobacillus leichmanii*; Booker & Stubbe, 1993) and so many types of class I enzymes?

The completion of the sequencing projects of the entire genome of *B. subtilis* and other bacteria will answer some of these questions. At present the finding that the *nrd* genes of *Myc. genitalium* are phylogenetically closer to the *nrdEF* genes of *B. subtilis* than to the *nrdAB* genes of *Enterobacteriaceae* confirms other observations indicating an evolutionary relationship between *Mycoplasma* and Gram-positive bacteria (Fraser *et al.*, 1995).

Expression of the *nrd* operon

The genetic analysis indicated that the coding sequence *ymaB* was essential. In contrast, inspection of the sequence between *nrdF* and *ymaB* revealed the presence of a stem-loop structure, which could act as an attenuator or terminator of transcription, and the presence of a putative σ A promoter. Therefore, we tested for the possible activity of the *nrdF*, *ymaB* intercistronic region, by cloning it upstream of a promoterless *lacZ* gene in the integrative plasmid pJM115. Strain PB1874, carrying the fusion in the *amyE* locus, did not synthesize β -galactosidase,

Fig. 4. β -Galactosidase assays of the *nrd-lacZ* fusion monitoring the induction of operon transcription by thymidine starvation. Half of the cultures in TM medium supplemented with thymidine ($50 \mu\text{g ml}^{-1}$) were collected at the indicated times (arrows), washed by centrifugation and resuspended in the same medium without thymidine. ■, wild-type strain; ●, wild-type strain starved of thymidine; ▲, *Rec⁻* strain; ▼, *Rec⁻* strain starved of thymidine.

suggesting the absence of a functional promoter in the intercistronic sequence (data not shown).

We propose that *ymaB* is co-transcribed with *ymaA*, *nrdE* and *nrdF*, and thus is part of the *nrd* operon.

To study the level of expression and to perform a preliminary analysis of the transcriptional control of the *nrd* operon, we used a *lacZ* transcriptional fusion, inserted into the chromosome downstream of *ymaB*. The fusion by means of plasmid pJM783/538 EB gave the results summarized in Table 3 and represented in Fig. 4. The level of transcription was low, but significant in rich and minimal media, and the presence of the mutations $\Delta\text{comP-A}$ and *recA-260* did not change the basal level of transcription significantly.

As in the case of the *nrdAB* operon of *E. coli* (Filpula & Fuchs, 1978), the transcription of RR genes increased as a consequence of a block of DNA synthesis obtained by thymidine starvation in the thymidine-requiring strain PB1850 (Fig. 4). Since this effect was higher in TM medium, a medium for the induction of competence in *B. subtilis* (Anagnostopoulos & Spizizen, 1961), we tested if

it was part of the competence response, and thus positively controlled by the response regulators ComP-A (Dubnau, 1993), or part of the RecA-controlled responses (Love & Yasbin, 1984).

In the $\Delta\text{comP-A}$ mutant strain PB1854, the peak of induction by thymidine starvation was comparable to that reached by the parental strain (Table 3). In contrast, the *recA* mutant PB1855 was severely affected in its ability to induce the operon after thymidine starvation (Fig. 4).

We can explain these results by the hypothesis that in *B. subtilis*, as in *E. coli* for *nrdAB*, the *nrdEF* operon is under the control of cell cycle and as a consequence under the influence of the SOS regulator RecA. In *B. subtilis*, RecA synthesis is induced both by thymidine starvation (Love & Yasbin, 1984) and by the attainment of the competence phase (Yasbin *et al.*, 1993). Since in the $\Delta\text{comP-A}$ mutant, thymidine starvation was still effective in inducing transcription of the *nrd* operon, we conclude that the increased level of expression of the operon is not due to the attainment of the competent state. The *nrd* induction can be tentatively classified as a Type I SOS phenomenon following the indication of Yasbin (1993).

These preliminary observations reinforce the suggestion that this is the functionally relevant operon in the synthesis of deoxyribonucleotides and is subject to fine control of expression to modulate their synthesis.

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REFERENCES

- Albertini, A. M. & Galizzi, A. (1985). Amplification of a chromosomal region in *Bacillus subtilis*. *J Bacteriol* **162**, 1203–1211.
- Albertini, A. M. & Galizzi, A. (1990). The *Bacillus subtilis outB* gene is highly homologous to an *Escherichia coli* *ntr*-like gene. *J Bacteriol* **172**, 5482–5485.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic Local Alignment Search Tool. *J Mol Biol* **215**, 403–410.
- Anagnostopoulos, C. & Spizizen, J. (1961). Requirements for transformation of *Bacillus subtilis*. *J Bacteriol* **81**, 741–746.
- Augustin, L., Jacobson, B. A. & Fuchs, J. A. (1994). *Escherichia coli* Fis and DnaA proteins bind specifically to *nrd* promoter region and affect expression of an *nrd-lac* fusion. *J Bacteriol* **176**, 378–387.
- Bazill, G. W. & Karamata, D. (1972). Temperature sensitive mutants in *B. subtilis* defective in deoxyribonucleotide synthesis. *Mol Genet* **117**, 19–29.
- Booker, S. & Stubbe, J. (1993). Cloning sequencing and expression of the adenosylcobalamin-dependent ribonucleotide reductase from *Lactobacillus leichmanii*. *Proc Natl Acad Sci USA* **90**, 8352–8356.
- Dubnau, D. (1993). Genetic exchange and homologous recombination. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 555–578. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

- Ferrari, E., Henner, D. J. & Hoch, J. (1981). Isolation of *Bacillus subtilis* genes from a Charon 4A library. *J Bacteriol* **146**, 430–432.
- Ferrari, F. A., Nguyen, A., Lang, D. & Hoch, J. A. (1983). Construction and properties of an integrable plasmid for *Bacillus subtilis*. *J Bacteriol* **154**, 1513–1515.
- Filpula, D. F. & Fuchs, J. A. (1977). Regulation of ribonucleoside reductase synthesis in *Escherichia coli*: increased enzyme synthesis as a result of inhibition of deoxyribonucleic acid synthesis. *J Bacteriol* **130**, 107–113.
- Filpula, D. F. & Fuchs, J. A. (1978). Regulation of the synthesis of ribonucleotide diphosphate reductase in *Escherichia coli*: specific activity of the enzyme in relationship to perturbations of DNA replication. *J Bacteriol* **135**, 429–435.
- Fisher, S., Rosenkrantz, M. S. & Sonenshein, A. L. (1984). Glutamine synthetase gene of *Bacillus subtilis*. *Gene* **32**, 427–438.
- Fleischmann, R. D. and others (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.
- Fraser, C. M. and others (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397–403.
- Gianni, M. & Galizzi, A. (1986). Isolation of genes preferentially expressed during *Bacillus subtilis* spore outgrowth. *J Bacteriol* **165**, 123–132.
- Gibert, I., Calero, S. & Barbé, J. (1990). Measurement of *in vivo* expression of *nrdA* and *nrdB* genes of *Escherichia coli* by using *lacZ* fusions. *Mol Gen Genet* **220**, 400–408.
- Hanahan, D. (1985). Techniques for transformation of *E. coli*. In *DNA Cloning: a Practical Approach*, vol. I, pp. 109–132. Edited by D. M. Glover. Oxford: IRL Press.
- Hanke, P. D. & Fuchs, J. A. (1983). Characterization of the mRNA coding for ribonucleoside diphosphate reductase in *Escherichia coli*. *J Bacteriol* **154**, 1040–1045.
- Hoch, J. A., Barat, M. & Anagnostopoulos, C. (1967). Transformation and transduction in recombination-defective mutants of *Bacillus subtilis*. *J Bacteriol* **93**, 1925–1937.
- Jordan, A., Gibert, I. & Barbé, J. (1994a). Cloning and sequencing of the genes from *S. typhimurium* encoding a new bacterial ribonucleotide reductase. *J Bacteriol* **176**, 3420–3427.
- Jordan, A., Pontis, E., Atta, M., Krook, M., Gibert, I., Barbé, J. & Reichard, P. (1994b). A second class I ribonucleotide reductase in *Enterobacteriaceae*: characterization of the *Salmonella typhimurium* enzyme. *Proc Natl Acad Sci USA* **91**, 12892–12896.
- Jordan, A., Argall, E., Gibert, I. & Barbé, J. (1996). Promoter identification and expression analysis of *Salmonella typhimurium* and *Escherichia coli* *nrdEF* operons encoding one of two class I ribonucleotide reductase present in both bacteria. *Mol Microbiol* **19**, 777–790.
- Karamata, D. & Gross, J. D. (1970). Isolation and genetic analysis of temperature sensitive mutants of *B. subtilis* defective in DNA synthesis. *Mol Gen Genet* **108**, 277–287.
- Kuroda, A., Asami, Y. & Sekiguchi, J. (1993). Molecular cloning of sporulation specific cell wall hydrolase gene of *Bacillus subtilis*. *J Bacteriol* **175**, 6260–6268.
- Love, P. E. & Yasbin, R. E. (1984). Genetic characterisation of the inducible SOS-like system of *Bacillus subtilis*. *J Bacteriol* **160**, 910–920.
- Nordlund, P. & Eklund, H. (1993). Structure and function of the *Escherichia coli* ribonucleotide reductase protein R2. *J Mol Biol* **232**, 123–164.
- Nordlund, P., Sjöberg, B.-M. & Eklund, H. (1990). Three-dimensional structure of the free radical protein of ribonucleotide reductase. *Nature* **345**, 593–598.
- Pearson, W. R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol* **183**, 63–98.
- Perego, M. (1993). Integrational vectors for genetic manipulations in *Bacillus subtilis*. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 615–624. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Perego, M., Ferrari, E., Bassi, M. T., Galizzi, A. & Mazza, P. G. (1987). Molecular cloning of *Bacillus subtilis* genes involved in DNA metabolism. *Mol Gen Genet* **209**, 8–14.
- Reichard, P. (1993). From RNA to DNA, why so many ribonucleotide reductases? *Science* **260**, 1773–1777.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scotti, C., Piatti, M., Cuzzoni, A., Perani, P., Tognoni, A., Grandi, G., Galizzi, A. & Albertini, A. M. (1993). A *Bacillus subtilis* large ORF coding for a polypeptide highly similar to polyketide synthases. *Gene* **130**, 65–71.
- Shimotsu, H. & Henner, D. J. (1986). Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene* **43**, 85–94.
- Sun, L. & Fuchs, J. A. (1992). *Escherichia coli* ribonucleotide reductase expression is cell cycle regulated. *Mol Biol Cell* **3**, 1095–1105.
- Sun, L., Jacobson, B. A., Dien, B. S., Srien, F. & Fuchs, J. A. (1994). Cell cycle regulation of the *Escherichia coli* *nrd* operon: requirement for a *cis*-acting upstream AT-rich sequence. *J Bacteriol* **176**, 2415–2416.
- Sun, X., Harder, H., Krook, M., Jorvall, H., Sjöberg, B.-M. & Reichard, P. (1993). A possible glycine radical in anaerobic ribonucleotide reductase from *Escherichia coli*: nucleotide sequence of the cloned *nrdD* gene. *Proc Natl Acad Sci USA* **90**, 577–581.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Uhlén, U. & Eklund, H. (1994). Structure of ribonucleotide reductase protein R1. *Nature* **370**, 533–539.
- Yang, F., Guizhen, L. & Rubin, H. (1994). Isolation of ribonucleotide reductase from *Mycobacterium tuberculosis* and cloning, expression and purification of the large subunit. *J Bacteriol* **176**, 6738–6743.
- Yasbin, R. E., Cheo, D. & Bol, D. (1993). DNA repair systems. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 529–537. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

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