Ribonucleotide reductase (RNR) of *Corynebacterium glutamicum* ATCC 13032 – genetic characterization of a second class IV enzyme

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Ribonucleotide reductases (RNRs) encoded by *nrd* (nucleotide reduction) genes are unique enzymes providing the DNA precursors in all living organisms and several viruses. The designation of four classes of RNRs reflects their use of diverse metallo-cofactors. Using oligonucleotide primers derived from conserved domains of the primary structure of known NrdA and NrdE proteins, an internal 938 bp fragment of the *nrdE* gene was amplified from genomic DNA of *Corynebacterium glutamicum*. With this PCR product a 4.36 kb fragment was identified and cloned containing the *nrdHIE* genes of *C. glutamicum*. A probe derived from *nrdF2* of *Mycobacterium tuberculosis* allowed the cloning and sequencing of the *nrdF* gene located 3.1 kb further downstream, encoding the small subunit of the *C. glutamicum* RNR. Conjugative introduction of *nrdE* from *C. glutamicum* complemented thermosensitive mutants of *Corynebacterium ammoniagenes* which had a defective catalytic subunit of the Mn-RNR. The authors provide arguments for allocation of the *C. glutamicum* NrdEF proteins to class IV in the RNR classification scheme of Stubbe & van der Donk (1995) [Chem Biol 2, 793–801].

Keywords: *Corynebacterium glutamicum*, *nrdHIE* genes, distal *nrdF* gene, heterologous genetic complementation of *nrd^{ts}* mutants, Mn-RNR of *C. ammoniagenes*

INTRODUCTION

It is now widely accepted that the biochemical evolution from the ancient RNA world to the present-day DNA world created a universal *de novo* route of DNA precursor biosynthesis (Follmann, 1982; Harder, 1993; Jordan & Reichard, 1998) for all living organisms, with the ribonucleotide reductases (RNRs) as key enzymes. These complex enzymes catalyse the irreversible deoxygenation of ribonucleoside 5'-di- or triphosphates to the corresponding 2'-deoxyribonucleotides concomitant

with the oxidation of a pair of thiol groups to disulfide in thioredoxin or other small proteins with redox-active thiols (Reichard, 1993). The observation that the same principle of allosteric regulation is encountered in all RNRs is a strong argument for the monophyletic origin of ribonucleotide reduction originally suggested by Follmann (1982). A radical mechanism is involved in this enzymic reaction and nature has combined the ability to reduce ribonucleotides with a diverse array of metallo-cofactors in different organisms in order to generate a transient protein radical that initiates nucleotide reduction (Stubbe & van der Donk, 1995). Thus trace elements like Co, Fe and Mn control growth and DNA production in diverse prokaryotic and eukaryotic organisms (Hogenkamp & McFarlan, 1984; Lammers & Follmann, 1983; Harder, 1993). The aerobic Fe-RNR of *Escherichia coli*, thought to represent the 'genuine' RNR of the *Enterobacteriaceae*, is the prototype of the class I enzymes consisting of the subunits R1 and R2 which are encoded by the genes *nrdAB* (Carlson *et al.*,

Abbreviations: *nrd*, nucleotide reduction; R1, large catalytic subunit of the RNRs encoded by *nrdA*; R2, small metal-containing subunit of the RNRs encoded by *nrdB*; R1E, large catalytic subunit of the RNRs encoded by *nrdE*; R2F, small metal-containing subunit of the RNRs encoded by *nrdF*; RNR, ribonucleotide reductase.

The GenBank accession numbers for the sequences reported in this paper are AF112535 for *nrdHIE* and AF112536 for *nrdF* of *C. glutamicum* ATCC 13032.

1984). The Mn-RNR of *Corynebacterium ammoniagenes* ($\alpha\beta_2$ holoenzyme; Willing *et al.*, 1988), represents the prototype of the class IV enzymes in the biochemical classification scheme of Stubbe & van der Donk (1995) and is designated as class Ib enzyme by Reichard (1993). The metallo-cofactor of this Mn-RNR contains a stable free radical (Gripenburg *et al.*, 1996) interacting only weakly with its intrinsic metal centre, a mononuclear, isolated Mn(II) site (Gripenburg, 1998). Independent studies confirmed that the active *C. ammoniagenes* Mn-RNR contains manganese and, in essence, lacks iron (Fieschi *et al.*, 1998). The *nrdEF* genes encoding the subunits R1E and R2F of this enzyme have been cloned recently (Fieschi *et al.*, 1998; Oehlmann *et al.*, 1998).

Coryneform bacteria *sensu stricto* and *sensu lato* (Liebl, 1992) are important industrial organisms for large-scale production of primary metabolites, namely amino acids and flavour-enhancing substances. The necessity of manganese deprivation as a prerequisite for inosine monophosphate production with *C. ammoniagenes* can be traced back (Auling & Follmann, 1994) to the Mn-RNR described above. However, the status of DNA precursor biosynthesis in the so-called glutamic acid bacteria (Kinoshita, 1985), well-known for large-scale production of amino acids, is not known. Merely, cell elongation of *Corynebacterium glutamicum* (and similar strains named *Brevibacterium divariaticum* and *B. lactofermentum* but now allocated to *C. glutamicum*) was reported to occur under conditions of Mn²⁺ deficiency (Oka *et al.*, 1968). This is reminiscent of the phenomenon of unbalanced growth death known from the closely related species *C. ammoniagenes*, which uses the Mn-RNR as class IV enzyme for its DNA precursor biosynthesis (Auling & Follmann, 1994).

In order to define the principles of manganese binding in the unusual Mn-RNR of coryneform bacteria on a broader basis the study of another class IV enzyme from this group of industrial micro-organisms was of interest. Here we report the cloning and sequencing of the *nrd* genes of *C. glutamicum* encoding the key enzyme of DNA precursor biosynthesis in this amino-acid-producing species.

METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. Strains were grown aerobically in Luria-Bertani broth containing 10 g tryptone, 5 g yeast extract and 5 g NaCl per litre (pH 7.2) at 120 r.p.m. and at 37 °C (*E. coli*) or 30 °C (corynebacterial strains). Strains harbouring plasmids were cultured in the presence of the selective antibiotic (50 µg l⁻¹).

DNA isolation and manipulation. Plasmid DNA was isolated according to the method of Birnboim & Doly (1979) and total DNA according to Ausubel *et al.* (1990). Corynebacteria were pretreated by shaking in lysis buffer containing 30 mg lysozyme ml⁻¹ for 1 h (plasmid DNA) or 3 h (total DNA) at 37 °C. DNA-manipulating enzymes were purchased from Boehringer Mannheim, BRL Life Technologies and Qiagen. DNA fragments were isolated from 0.8% agarose gels using the QiaexII kit (Qiagen). Southern hybridizations were carried out by

standard methods (Sambrook *et al.*, 1989). Labelling of probes with digoxigenin-coupled dUTP (Boehringer) was done by PCR.

PCR amplification of the probes used for cloning. For amplification of an internal part of the *nrdE* gene of *C. glutamicum*, degenerate primers (Biometra) were designed from two conserved peptide sequences (SNLCXEI; LYYXR) of the NrdA and NrdE proteins, respectively (Fig. 1), namely primer P1, 5'-AGC AAC CTG TGC TCG GAG AT-3', and primer P2, 5'-CGC AGC CGG ATG TAG TAC AG-3'. *Taq* polymerase from Appligene Oncor was used for PCR. Samples of 50 µl, overlaid with the same volume of paraffin oil, were prepared according to the manufacturer's instructions. Before adding *Taq* polymerase the mixture was heated at 94 °C for 5 min followed by 30 cycles of the following programme: 1 min at 94 °C, 45 s at 45 °C and 1 min at 70 °C. In order to generate a probe for the gene of the small subunit of *C. glutamicum* the *nrdF* genes of *Mycobacterium tuberculosis* were amplified with the above PCR protocol but at an increased annealing temperature of 51 °C. The primers used were P3 (5'-ATG ACC GGC AAG CTC GT-3') and P4 (5'-GAA GTC CCA GTC GGT GT-3') for *nrdF1*, and P5 (5'-GTG ACT GGA AAC GCA AA-3') and P6 (5'-GAA GTC CCA GTC ATC GT-3') for *nrdF2*, respectively.

Screening of a *C. glutamicum* cosmid library and cloning of the *nrd* genes. For cloning of the *nrd* genes of *C. glutamicum* a cosmid library containing chromosomal DNA fragments of *C. glutamicum* ATCC 13032 with a size of 40 kb was used (Bathe *et al.*, 1996). A total of 576 clones were grown in numerical arrangement as single colonies on Nytran membranes (Schleicher & Schuell) placed on LB agar. Positive clones were identified by colony hybridization (Sambrook *et al.*, 1989) using the probes described above. After digestion with the restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I and *Xba*I the cosmid named 810 was chosen for final cloning.

Sequencing and sequence analysis. Plasmid pWCG3 containing the *nrdHIE* genes of *C. glutamicum* was separately digested with *Eco*RI and *Pst*I. The isolated fragments of the 4.36 *Xba*I-*Bam*HI insert were subcloned into pUC18, resulting in plasmids pWCG2, pWCG32, pWCG35 and pWCG36 (Table 1). In order to sequence *nrdF* the two positive *Pst*I fragments (0.93 and 1.31 kb) obtained after Southern blotting were cloned into pUC18. The two resulting plasmids were named pWCG51 and pWCG52 (Table 1, Fig. 3). Standard primers were used for the sequencing reaction. Primer walking allowed closure of gaps in the double-strand sequence of *nrdHIE* and *nrdF*. DNA was sequenced with the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) using an ABI Prism 310 Genetic Analyzer. The BLAST e-mail server at the National Center for Biotechnology Information (NCBI) was used for comparison of sequences.

Genetic complementation of mutants. In order to complement the *nrdE*^{ts} mutants CH31 and CH33 derived from *C. ammoniagenes* (Luo *et al.*, 1997) with the *nrdE* gene of *C. glutamicum*, the 4.36 kb *Xba*I-*Bam*HI fragment was excised from pWCG3 and ligated to the *E. coli*-*C. glutamicum* shuttle vector pECM2 (Schäfer *et al.*, 1990). The resulting plasmid pWCG6 was transformed into the mobilizing *E. coli* strain S17-1 by the method of Sambrook *et al.* (1989). This donor was then used in conjugation experiments exactly as described by Schäfer *et al.* (1990). Prior to mating, the recipients CH31 and CH33 were treated for 9 min at 48 °C in order to enhance the number of transconjugants (Schäfer *et al.*, 1994). After antibiotic selection, single colonies of transconjugants were

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Bacteria		
<i>C. glutamicum</i> ATCC 13032	Wild-type	American Type Culture Collection
<i>C. glutamicum</i> R163	Restriction-deficient mutant of <i>C. glutamicum</i> ATCC 13059	Liebl & Schein (1990)
<i>C. ammoniagenes</i> ATCC 6872	Wild-type	Laboratory stock
<i>C. ammoniagenes</i> CH31 and CH33	<i>nrdE</i> ^{ts} mutants of ATCC 6872	Luo <i>et al.</i> (1997)
<i>E. coli</i> S17-1	Mobilizing donor strain, <i>pro recA</i> , which has an RP4 derivative integrated into the chromosome	Simon <i>et al.</i> (1983)
<i>E. coli</i> XL1Blue	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173$	Jerpseth <i>et al.</i> (1993)
Plasmids		
pUC18/19	Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pECM2	Mobilizable <i>E. coli</i> - <i>Corynebacterium</i> shuttle vector, Km ^r Cm ^r	Schäfer <i>et al.</i> (1990)
pWCG2	pUC18 with 1.25 kb <i>EcoRI</i> insert	This work
pWCG3	pUC18 with 4.36 kb <i>XbaI</i> - <i>BamHI</i> insert	This work
pWCG32	pUC18 with 1.87 kb <i>EcoRI</i> insert	This work
pWCG35	pUC18 with 1.03 kb <i>PstI</i> insert	This work
pWCG36	pUC18 with 1.16 kb <i>PstI</i> insert	This work
pWCG51	pUC18 with 0.93 kb <i>PstI</i> insert	This work
pWCG52	pUC18 with 1.31 kb <i>PstI</i> insert	This work
pWCG6	pECM2 with 4.36 <i>XbaI</i> - <i>BamHI</i> insert	This work

picked and spread onto two plates for a 2 d incubation at 30 °C and 37 °C, respectively.

RESULTS

Cloning the genes *nrdHIE* and *nrdF* of *C. glutamicum*

Our cloning strategy was based on the assumption that the genes of the two subunits of the *C. glutamicum* RNR would be located in close proximity, as in other bacterial *nrd* operons known when our study was initiated (Carlson *et al.*, 1984; Jordan *et al.*, 1994). A comparison of the primary structures of known NrdA and NrdE proteins revealed at least five conserved regions. The two most striking regions were used for the design of the primers P1 and P2 (Fig. 1). With these two primers it was

possible to amplify a fragment of 938 bp from chromosomal DNA of *C. glutamicum*. The size of the amplicate was as expected from the positions of the primers within the *nrd* genes. This PCR product was sequenced and compared to known sequences in the databases (GenBank). The best findings were 75 % identical bases with *nrdE* of *Mycobacterium tuberculosis* and 70 % with *nrdE* of *Salmonella typhimurium*. High similarity was also observed in the deduced amino acid sequence. This allowed us to conclude that the 938 bp fragment is in fact a part of a *nrdE* gene of *C. glutamicum*. Next, the PCR fragment was used as a probe for screening a cosmid library of *C. glutamicum* (Bathe *et al.*, 1996). The four positive out of 576 cosmid clones were submitted to restriction analysis and the cosmid 810 was

Fig. 1. Design of degenerate primers for cloning the *nrd* genes of *C. glutamicum* from two highly conserved regions of known NrdA and NrdE proteins. The sequences of the primers were copied from the nucleotide sequence corresponding to the conserved regions, considering the codon usage of coryneform bacteria (Eikmanns, 1992) for variable positions.

Fig. 2. Southern blots of cosmid 810 with the probes for the detection of *nrdE* (a) and *nrdF* (b) of *C. glutamicum* after digestion with different restriction endonucleases. The *nrdE*-containing fragments (a) obtained after digestion with *Bam*HI, *Sal*I and *Xba*I were used in comparison with sequence data for a rough calculation of the distance of *nrdF* to *nrdHIE*. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xba*I.

Fig. 3. Restriction map of the *nrd* locus of *C. glutamicum* ATCC 13032. The arrows a, b, c and d indicate the location and direction of the primers used for the determination of the distance of *nrdF* to *nrdHIE*. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xba*I.

chosen for final cloning. From its 40 kb insert, restriction fragments were identified by Southern hybridization with the above PCR probe (Fig. 2a). Finally, a 4.36 kb fragment obtained by restriction with *Xba*I and *Bam*HI was ligated to pUC18, resulting in plasmid pWCG3 (Fig. 3), and used for sequencing. Analysis of its sequence showed that the complete *nrdE* gene encoding the large catalytic subunit R1E of the *C. glutamicum* RNR was present on this 4.36 kb *Xba*I–*Bam*HI fragment. Two additional open reading frames, orientated in the same direction as *nrdE*, were identified as *nrdH* (234 bp) and *nrdI* (447 bp). However, the gene encoding the R2F subunit of the *C. glutamicum* RNR was not found on the 4.36 kb *Xba*I–*Bam*HI insert of plasmid pWCG3 (Fig. 3).

A new tool for cloning of the missing gene was therefore required. Based on the high similarity between the *nrdE* genes of *C. glutamicum* and *M. tuberculosis* (see Table 2) it was expected that the *nrdF* genes of the two species would also be highly similar, and we decided to use the *M. tuberculosis* *nrdF* gene as a hybridization probe. Yang *et al.* (1997) have described two *nrdF* genes in *M.*

tuberculosis. Only the probe derived from *nrdF2* hybridized with chromosomal DNA of *C. glutamicum* and was used for cloning (see Methods) of the identified fragments (Fig. 2b).

The exact distance between these genes and the orientation of *nrdF* to the other three *nrd* genes remained unknown because no common fragment for *nrdHIE* and *nrdF* was detected by Southern blotting (Fig. 2). This objective was achieved by bridging the gap between the genes of interest using PCR. For this purpose oligonucleotide primers were derived from sequences located at the ends of the 4.36 kb *Xba*I–*Bam*HI fragment and in the middle of the *nrdF* gene (Fig. 3) in order to cope with the four possible arrangements. Using cosmid 810 as template for PCR only the reaction combining primers b and d (Fig. 3) allowed the amplification of a fragment. Restriction analysis of this 3.2 kb product revealed the presence of all expected restriction sites known from sequence data. Based on this finding we calculated that *nrdF* is located 3.1 kb downstream of *nrdE* in the same orientation (Fig. 3). Thus, cosmid 810 was the only one containing the four genes *nrdHIEF* of *C. glutamicum*.

Table 2. Percentages of identical amino acids in NrdE/F of *C. glutamicum*, *C. ammoniagenes*, *M. tuberculosis*, *E. coli*, *S. typhimurium*, *M. genitalium* and *B. subtilis*, and NrdA/B of *E. coli*

The data represent the percentage of identical amino acids within the NrdE/NrdF proteins. The following sequences were obtained from GenBank: *Bacillus subtilis* (B.s.), Z68500; *C. ammoniagenes* (C.a.), Y09572 and AF050168; *C. glutamicum* (C.g.), AF112535 and AF112536 (this work); *E. coli* (E.c.), AE000352; *M. tuberculosis* (M.t.), U41099 and U41100; *Mycoplasma genitalium* (M.g.), U39701; *Salmonella typhimurium* (S.t.), X73226; *E. coli* (E.c.) NrdAB, AE000313.

Species	C. g.	C. a.	M. t.	E. c.	S. t.	M. g.	B. s.	E. c. NrdAB
<i>C. glutamicum</i>	100/100	82/79*	78/74	72/66	70/65	56/49	48/42	27/27
<i>C. ammoniagenes</i>		100/100	74/73	69/64	68/64	54/54	46/42	26/26
<i>M. tuberculosis</i>			100/100	71/71	70/71	57/51	46/43	27/28
<i>E. coli</i>				100/100	89/88	54/51	42/44	26/26
<i>S. typhimurium</i>					100/100	54/50	44/45	25/27
<i>M. genitalium</i>						100/100	43/34	27/28
<i>B. subtilis</i>							100/100	25/26
<i>E. coli</i> NrdAB								100/100

In order to answer the question whether *nrdE* and *nrdF* exist only as a single copy on the *C. glutamicum* chromosome, total DNA of *C. glutamicum* was examined in additional hybridization studies with the original probes for cloning (938 bp PCR product, *nrdF2* of *M. tuberculosis*). Only one positive signal was observed for each gene probe even under non-stringent conditions.

DNA sequence analysis of the *nrdHIEF* genes

The 4363 bp insert of plasmid pWCG3 (Fig. 3) contains three open reading frames in the order *nrdH* (234 bp), *nrdI* (447 bp) and *nrdE* (2124 bp). The *nrdF* gene (1005 bp) is located 3.1 kb downstream of *nrdE* on the genome of *C. glutamicum* ATCC 13032. The G+C content of the *nrd* genes (*nrdH* 57.3 mol%; *nrdI* 49.9 mol%; *nrdE* 54.0 mol%; *nrdF* 51.7 mol%) is within the range described for genes of corynebacterial origin (Malumbres *et al.*, 1993). Likewise, the codon usage of the *nrdHEF* genes is in accordance with that reported for other genes of *C. glutamicum* (Eikmanns, 1992). An exception is the codon usage in *nrdI* because AT-rich codons are preferred for the amino acids Asp, Gln, Lys, Phe and Tyr.

Starting signals for translation were identified for all four *nrd* genes. Either 9 bp or 11 bp upstream of the ATG start codons of the genes *nrdHIEF* there are purine-rich octamers (AAGGGAAG) which fit well to the 3' end of the 16S rRNA of *C. glutamicum* (GenBank Y12792) and may represent the Shine–Dalgarno sequence of the ribosome-binding site (RBS). The RBS of *nrdE* probably contains the pentamer GGGAA as Shine–Dalgarno sequence located 8 bp upstream of the start codon GTG. A possible promoter region with a –10 sequence (TTTAAA) and a –35 sequence (TTTGTT) was found

between positions 125 and 161 of the insert of pWCG3 (GenBank AF112535). Both sequences are rather similar to the consensus promoter sequence of *C. glutamicum* (Pátek *et al.*, 1996). Upstream of *nrdI* and *nrdE* no promoter sequences were identified. This indicates that the three genes are transcribed as a unique polycistronic mRNA which is terminated by a palindromic region of 41 bp located 7 bp downstream of the stop codon of *nrdE*. A putative promoter of the *nrdF* gene with a –10 sequence (TCTAAA) and a –35 region (TTTTGG) was found between the positions 282 and 308 of the *nrdF* (GenBank AF112536) but no signal for transcriptional termination.

The deduced amino acid sequences of the *nrdE* and the *nrdF* genes from *C. glutamicum* strongly resemble those of NrdE and NrdF of *C. ammoniagenes* and *M. tuberculosis*. The percentages of identical amino acids are listed in Table 2 and clearly discriminate the *nrd* genes of *C. glutamicum* from the *nrdAB* system of *E. coli* encoding the prototype of the class I enzymes. The DNA sequence of the *nrdE* and the *nrdF* genes encoding the two subunits of the *C. glutamicum* RNR allowed us to calculate a molecular mass of 74.0 kDa from the 707 amino acid residues of the NrdE protein (GTG as start codon) and of 34.36 kDa from the 334 residues of NrdF.

Heterologous genetic complementation

The availability of temperature-sensitive *nrdE* mutants of *C. ammoniagenes* ATCC 6872 (Luo *et al.*, 1997) offered the attractive possibility to demonstrate heterologous genetic complementation of these mutants with the *nrdE* gene cloned from *C. glutamicum*. The *nrdE*^{ts} strains CH31 and CH33 cannot grow at 37 °C due to a defective large catalytic subunit of the Mn-RNR but are

Fig. 4. Growth of the *nrdE*^{ts} mutants CH31 and CH32 of *C. ammoniagenes* after genetic complementation with pWCG6 containing *nrdE* of *C. glutamicum* by RP4-mediated conjugation (Schäfer *et al.*, 1990). The cells were incubated at 27 °C (a) and 37 °C (b) for 2 d. A1, *C. ammoniagenes* wild-type/pECM2; B1, *C. glutamicum* wild-type/pECM2; C1, CH31/pECM2; C2–5, CH31/pWCG6; D1, CH33/pECM2; D2–5, CH33/pWCG6.

able to grow under permissive conditions (27 °C). Upon conjugational transfer of plasmid pWCG6, which contains the *nrdE* gene of *C. glutamicum*, all transconjugants of CH31 and CH33 were able to grow at 37 °C although with a lower growth rate than the wild-type. Transconjugants which had received the vector only did not grow at this non-permissive temperature (Fig. 4). The plasmids were reisolated from the complemented transconjugants and the control. Restriction analysis confirmed that pWCG6 was only present in those cells which were able to grow at 37 °C. Thus, restoration of growth in both *nrdE*^{ts} mutants of *C. ammoniagenes* ATCC 6872 is caused by the *nrdE* gene of *C. glutamicum*.

DISCUSSION

A divergent pattern of genomic organization is emerging in the Gram-positive bacteria from the increasing sequence information on chromosomal regions harbouring the *nrdEF* genes obtained either from direct cloning (Jordan *et al.*, 1996; Yang *et al.*, 1994, 1997) or from genome sequencing projects (Fraser *et al.*, 1995; Scotti *et al.*, 1996; Himmelreich *et al.*, 1996; Blattner *et al.*, 1997; Cole *et al.*, 1998). At present five different arrangements of the *nrd* region are known (Fig. 5). Upon a broader view the basic underlying pattern corresponds well to the phylogenetic division of the Gram-positive bacteria into a low- and a high-GC branch. In the first group the *nrdEF* genes are located on a contiguous stretch of DNA (Scotti *et al.*, 1996; Jordan *et al.*, 1996), even when rearranged by an unknown event as in *Mycoplasma pneumoniae* (Himmelreich *et al.*, 1996). In the latter, now elevated to class rank as *Actinobacteria* (Stackebrandt *et al.*, 1997), *nrdF* is distinctly separated from *nrdE* with a gap increasing in the order *C. ammoniagenes* (1.2 kb, Fieschi *et al.*, 1998; Oehlmann, 1998), *C. glutamicum* (3.1 kb, this work) and *Mycobacterium*

tuberculosis (1186 kb, Cole *et al.*, 1998). The case of *M. tuberculosis* appears exceptional because this pathogen has a functional and a non-functional copy of the *nrdF* gene (Yang *et al.*, 1997).

The marked distance separating the genes *nrdHIE* and *nrdF* in *C. glutamicum* disproved our initial working hypothesis that both genes encoding the RNR of this species were easily accessible from a common genomic DNA fragment and in fact considerably delayed cloning of the gene of the small subunit. More lucky was our choice of primers for cloning the gene of the large catalytic subunit of RNR from *C. glutamicum* based on the fundamental assumption (Follmann, 1982) that the domains used here reflect a monophyletic origin of the substrate reduction and also a polyphyletic origin of the diverse radical-generating system (including subunits, also referred to as metallo-cofactors: Stubbe & van der Donk, 1995). For design of cloning primer P1 the region with the highest conservation of protein primary structure was exploited. The functionally important region SNLCXEI contains the essential cysteine residue, proposed to generate the radical which initiates the substrate reduction (Uhlen & Eklund, 1994), and appears as a perfect target to design primers for cloning RNR genes. The region (LYYXR) used for design of primer P2 exists only in NrdA and NrdE proteins (Oehlmann, 1998) and strongly reflects the evolutionary adaptation of the class I and IV RNRs to the use of protein-bound metal for the generation of a stable radical. The two tyrosyl residues present in this region connect the long-range electron transfer from the small radical-containing subunit to the active centre in the large catalytic subunit (Ekberg *et al.*, 1996). In addition, the primer design for the two regions of interest was facilitated by the codon usage in corynebacteria. These organisms prefer the GC-rich codons, especially for amino acids with only two possible codons (Eikmanns, 1992). Their frequency explains why the cloning primers used here (Fig. 1) had only three and four mismatches, respectively.

The function of the two small ORFs preceding *nrdE* in *C. glutamicum* (this work) and in *C. ammoniagenes* (Fieschi *et al.*, 1998) is not yet clear. The amino acid sequence deduced from the *nrdH* gene (77 residues, 8 kDa) shows high similarity to NrdH of *E. coli*, *Salmonella typhimurium* and *Lactococcus lactis* and may function as a glutaredoxin, a specific electron donor, for the RNRs composed of NrdE and NrdF (Jordan *et al.*, 1996; 1997). The hypothetical NrdI protein consists of 148 amino acid residues with a predicted molecular mass of 16.4 kDa but an unknown function in ribonucleotide reduction. Recently, in *S. typhimurium* a stimulatory effect of NrdI on the ribonucleotide reduction catalysed by NrdEF was reported (Jordan *et al.*, 1997).

The sequence similarity of the *nrdEF* genes within the Gram-positive bacteria parallels the phylogenetic branching (Fig. 6), i.e. the percentages of identical amino acids in the NrdE and the NrdF proteins of *C. glutamicum* are very high between members of the high-

Fig. 5. Diverse arrangement of the *nrdEF* loci in Gram-positive bacteria. Taking *nrdH* and *nrdI* into account will additionally increase the diversity of the *nrd* loci. C.g., *C. glutamicum* (this work); C.a., *C. ammoniagenes* (Fieschi *et al.*, 1998; Oehlmann *et al.*, 1998); M.t., *M. tuberculosis* (Yang *et al.*, 1994, 1997); M.p., *Mycoplasma pneumoniae* (Himmelreich *et al.*, 1996); B.s., *Bacillus subtilis* (Scotti *et al.*, 1996); L.I., *Lactococcus lactis* (Jordan *et al.*, 1997).

Fig. 6. Phylogenetic tree derived from known NrdF proteins and NrdB of *E. coli* as outlying sequence. CLUSTAL W (version 1.5b) was used for sequence alignment. The tree was generated with 1000 bootstrap trials and the bootstrap values are labelled at the nodes. Sequence information was obtained from GenBank: *Bacillus subtilis* (P50621), *C. ammoniagenes* (AF050168), *C. glutamicum* (AF112536 for *nrdF*; this work), *E. coli* (AE000352), *Mycobacterium tuberculosis* (*nrdF1* U41099; *nrdF2* U41100), *Mycoplasma genitalium* (V39701), *Mycoplasma pneumoniae* (AE000050), *Salmonella typhimurium* (X73226) and *E. coli* *nrdB* (AE000313).

GC branch, and lower when compared to members of the low-GC branch (Table 2). The exceptionally high value of identical amino acids in the NrdEF proteins of *C. glutamicum*, *E. coli* (69%, Blattner *et al.*, 1997) and *S. typhimurium* (70%, Jordan *et al.*, 1994) suggests a horizontal gene transfer of the *nrdEF* genes from the *Actinobacteria* to a common ancestor of *E. coli* and *S.*

typhimurium when taking into account that these genes are absent in the closely related *Haemophilus influenzae* (Fleischmann *et al.*, 1995).

The alignment of NrdF from *C. glutamicum* and *C. ammoniagenes* with NrdB of *E. coli* (Fig. 7) reveals that the functional constraints for the evolution of the metal and radical containing subunit have conserved specific domains. Thus, the tyrosyl residue in position 120 of the *C. glutamicum* NrdF equivalent to the Tyr₁₁₄ of the *C. ammoniagenes* NrdF (Oehlmann *et al.*, 1998) corresponds to the Tyr₁₂₂ bearing the organic radical in the *E. coli* Fe-RNR (Nordlund *et al.*, 1990). This alignment suggests that both corynebacterial NrdF proteins contain a tyrosyl radical. In addition, the constraints of the radical mechanism required for reduction of ribonucleotides appear to be very strong in both the Fe- and the Mn-enzyme, i.e. the three residues building a hydrophobic pocket probably necessary for the stabilization of the radical in the Fe-RNR (Ormö *et al.*, 1995) are also present in the NrdF proteins of *C. ammoniagenes* and *C. glutamicum*. Likewise, all amino acids proposed to be involved in the long-range electron transfer through the NrdB protein are conserved in the corynebacterial proteins (Fig. 7) as are the residues responsible for binding the iron in the *E. coli* RNR (Nordlund *et al.*, 1990; Sjöberg, 1997). All these probably functionally important residues are present not only in the corynebacterial RNRs but also in all known NrdEF proteins with the exception of the genus *Mycoplasma*. In comparison to the primary structure of NrdB there are several gaps in the amino acid sequence of the NrdF proteins. Modelling of the corynebacterial NrdF proteins (Oehlmann, 1998) reveals that these deletions occur in the peripheral shell and do not change the coordination of the interior α -helices containing the conserved residues, which bind the iron in the NrdB protein (Nordlund *et al.*, 1990). However, the sequence alignment does not allow inference of the metal specificity of the *C. glutamicum* RNR. Recent biochemical data from ICP-MS measurements of R2F from *C. glutamicum* revealed that the active protein contains manganese but no iron (Abbouni *et al.*, 1999). This supports the assignment of the *C. glutamicum* enzyme to class IV of the RNR classification scheme of Stubbe & van der Donk (1995). Thus, a second manganese enzyme is added to the *C. ammoniagenes* RNR, which clearly contains manganese (Willing *et al.*, 1988; Gripenburg *et al.*, 1998; Fieschi *et al.*, 1998). In this work hybrid Mn-RNRs were generated *in vivo* by the successful genetic complementation between the resident *nrdF* gene in the *C. ammoniagenes* *nrdE*^{ts} mutants and the *nrdE* gene introduced from the *C. glutamicum* wild-type, indicating the cooperation of the heterologous subunits. This contrasts with the previous failure to establish heterologous biochemical complementation with combinations of the *C. ammoniagenes* Mn-RNR and the *E. coli* Fe-RNR (Willing *et al.*, 1988).

A possible major role for the residues in the second coordination sphere determining the metal specificity of the RNRs has been extensively discussed by Fieschi *et al.*

C.g.	--MAADSDLSVHDAYLKEHVAPVKAINWNSIPDSKDLEVWDRLTGNFWLPEKVPVSNDIKSWG	62
C.a.	-----SNEYDEYIANHTDPVKAINWNVIPDEKDLEVWDRLTGNFWLPEKIPVSNDIQSWNK	56
M.t.	-----MTGNAKLIDRVSAINWNRLQDEKDAEVWDRLTGNFWLPEKVPVSNDIPSWG	52
E.c.B	AYTTFSQTKNDQLKEPMFFGQPVNVARYDQKYDIFEKLEKQLSFFWRPEEVDVSRDRIDYQA	64
↓ ↓ ↓		
C.g.	LNEVEKAATMRVFTGLTLIDTIQGTVGAISSLPPDADSLHEEAVLTNIAFMESVHAKSYSNIFMT	126
C.a.	MTPQEQLATMRVFTGLTLIDTIQGTVGAISSLPDVETMHEEGVYTNIAFMESVHAKSYSNIFMT	120
M.t.	LTAGEKQLTMRVFTGLTMDTIQGTVGAVSLIPDALTPHEEAVLTNIAFMESVHAKSYSQIFST	116
E.c.B	LPEHEKHIFISNLKYQTLIDSIQGRSPNVALLPLISIPETLWVETWAFSTLHRSYTHIIRN	128
↓ ↓ ↓		
C.g.	LASTAEINDAFRWSEENENLQRKAKIILSYEGDDP-----LK	164
C.a.	LASTPQINEAFRWSEENENLQRKAKIIMSYYNGDDP-----LK	158
M.t.	LCSTAEIDDAFRWSEENRNLQRKAEIVLQYRYGDEP-----LK	154
E.c.B	IVNDPSV--VFDDIVTNEQIQKRAEGISSYDELIEMTSYWHLLGEGTHTVNGKTVTVSLRELK	190
↓ ↓ ↓		
C.g.	RKI-----ASVILLESFLFYSGFYLPYMWSSHKLNTADVIRLIIRDEAVHGYIIGYKYQKAVA	223
C.a.	KKV-----ASTLILESFLFYSGFYLPYMLSSRAKLNTADIIRLIIRDESVMGYIIGYKYQQGVK	317
M.t.	RKV-----ASTLILESFLFYSGFYLPYMWSSRAKLNTADMIRLIIRDEAVHGYIIGYKFGRLA	213
E.c.B	KKLYLCLMSVNAIEAIRFFYVSFACSFAPAEERELMEGNAKIRLIARDEAVHGYIIGYKFLNLLRS	254
↓ ↓ ↓		
C.g.	KETP----ERQEELKEYTFDLLYDLYDNETQYSEDLYDD---LGWTEDEVK-RFLRYIANKALNN	280
C.a.	KLSE----AEQEEYKAYTFDLMYDLYENEIEYTEDIYDD---LGWTEDEVK-RFLRYNANKALNN	274
M.t.	LVDD----VTRAEKDYTYELLFELYDNEVEYTTQDLYDE---VGLTEDEVK-KFLRYNANKALMN	270
E.c.B	GADDPEMAEIAEECKQECYDLFVQAAQKEKDWADYLFRDGSMIGLNKDILCQYVEYITNIRMQA	318
↓		
C.g.	LGYEGLFPADETKVSPNLSALSPNADENHDFSGSGSSYVIGKAENTEDDDWDF----	334
C.a.	LGYEGLFPTDETUKVSPAILSSLSNADENHDFSGSGSSYVIGKAEDTTDDWDF----	328
M.t.	LGYEALFPRDETVPNPAISALSPNADENHDFSGSGSSYVIGKAVTDDDDWDF----	324
E.c.B	VGLDLFPOTR--SNPIPWINTWLVSDNVQVAPQEVVSSYLVGQIDSEVDTDDLNSNFQL	375

Fig. 7. Alignment of NrdF proteins of *C. glutamicum*, *C. ammoniagenes* and *M. tuberculosis* with NrdB of the Fe-RNR of *E. coli*. The tyrosine residue probably carrying the organic radical is in bold. The proposed metal ligands are boxed. The downward-pointing arrows indicate amino acids involved in the electron transfer; residues of the hydrophobic pocket thought to stabilize the radical are labelled with an exclamation mark (!). For accession numbers see the legend to Fig. 6.

(1998) based on observations with other manganese enzymes, cambialistic or not (Beyer *et al.*, 1991; Graeff-Wohlleben *et al.*, 1997; Gabbianelli *et al.*, 1997; Boldt *et al.*, 1997). Such a concept implies that the metal centre of the corynebacterial NrdF proteins has not been a direct target of the evolutionary drift ultimately leading to the divergence of the *nrdB* and *nrdF* genes. This view may change with advancing knowledge of the direct ligands in the metal centre of the unusual RNRs of *C. glutamicum* and *C. ammoniagenes*.

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