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S434F in NrdE Generates the Thermosensitive Phenotype of *Corynebacterium ammoniagenes* CH31 and Enhances Thermolability by Increasing the Surface Hydrophobicity of the NrdE(Ts) Protein

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The thermosensitive phenotype of strain CH31, a derivative of *Corynebacterium ammoniagenes* ATCC 6872, was allocated by cloning, sequencing, and genetic complementation to a single C→T exchange in the *nrdE* (*n*ucleotide *red*uction) gene at nucleotide 1301. Protein modeling indicates the impaired surface hydrophobicity of NrdE(Ts) due to the S434F transition.

A unique enzyme, ribonucleotide reductase (EC 1.17.4.1), delivers the building blocks for DNA replication in all living organisms in a potentially rate-limiting step (9, 29, 34, 38). In the gram-positive nucleotide producer (2, 4, 6) *Corynebacterium* (formerly *Brevibacterium*) *ammoniagenes*, this essential enzyme is a manganese protein (Mn-ribonucleotide reductase) encoded by the *nrdEF* (*n*ucleotide *red*uction) genes, and the enzymatically active holoenzyme has an $\alpha\beta_2$ subunit structure (5, 10, 24, 25, 44). By random chemical mutagenesis of *Corynebacterium ammoniagenes* ATCC 6872 using *N*-methyl-*N*′-nitro-*N*-nitrosoguanosine (MNNG), temperature-sensitive (TS) clones which failed to reduce ribonucleotides to 2′-deoxyribonucleotides were obtained (18). Through in vitro complementation, their defect was located biochemically on the CA1 protein, encoded by the *nrdE* gene (18, 24), and therefore renamed R2E (NrdE). Recently, one of these TS mutants, strain CH31, was exploited for nucleotide overproduction by temperature shift in the presence of excess manganese (1).

Here we characterize the *nrdE* gene of *C. ammoniagenes* CH31 with respect to the putative point mutation expected (33) from the foregoing mutagenization with the alkylating compound MNNG.

First, the *nrdE* genes of CH31 and its parental wild type, *C. ammoniagenes* ATCC 6872, were cloned from cetyltrimethylammonium bromide-extracted (19) chromosomal DNA into XmaI-digested pUC18 and, after ligation, transformed into *Escherichia coli* XL1-Blue. Strains of *C. ammoniagenes* were routinely cultivated in seed medium (18) at 27°C and *E. coli* XL1-Blue at 37°C in LB (25) supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), D-glucose (0.5%, wt/vol), or IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM), as required. A 0.5-kb fragment of the *nrdE* gene from *C. ammoniagenes* ATCC 6872 (Fig. 1) was amplified and digoxigenin labeled (16) by PCR using primers 5′-GGCCAGAGAA CCTCCACGGC-3′ (forward) and 5′-TTGTCCATGTGTGG AGCTG-GG-3′ (backward) for specific probing (30) of the two chromosomal 5.2-kb XmaI fragments and the recombinant plasmids pUCEF6872 and pUCEFCH31 (Table 1). A 2.3-kb region of the *nrd* operon of *C. ammoniagenes* was sequenced (Fig. 1) by a primer walking approach and the chain termination method (31) using a BigDye Terminator cycle sequencing kit on an ABI Prism 310 genetic analyzer (PE Applied Biosystems Inc.). The complete *nrdE* gene, including the intergenic region upstream and 100 bp downstream, was analyzed using the DNASTAR software package (DNASTAR Inc., Madison, WI) and Clone Manager 5.0 (Scientific & Educational Software) and CLUSTAL W for multiple sequence alignments (39). Sequence comparison between the wild type (accession no. AY769914) and mutant (accession no. AY788949) revealed only a single base pair substitution, at position 1301 (C→T), within the *nrdE* coding region, causing a serine-to-phenylalanine exchange at position 434 in NrdE (S434F). Only when we compared the sequence to one deposited *nrdE* gene sequence (accession no. Y09572 [8]) did three additional base

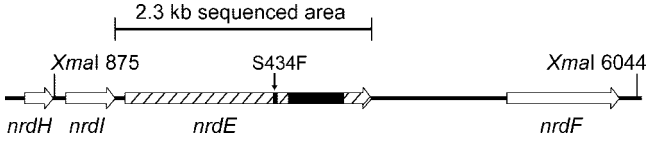

FIG. 1. Restriction map of the *nrd* locus of *C. ammoniagenes* strains ATCC 6872 (24, 40) and CH31 (7). The arrow indicates the point mutation within the *nrdE* gene. The position of the *nrdE* hybridization probe is shown as a black bar.

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TABLE 1. Microorganisms and plasmids

Microorganism	Related genotype, phenotype, or feature	Source/reference
Strains		
Corynebacterium ammoniagenes	ATCC 6872, wild type, *nrdE*$^+$	Institute's culture collection
Strain CH31	Thermosensitive mutant of ATCC 6872, *nrdE*(Ts)	18
Escherichia coli XL1-Blue	*endA1 gyrA96 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44 thi-1* F′(*proAB lacI*^{q}*ZΔM15* Tn*10*)	Stratagene
Plasmids		
pUC18	Amp^r, cloning vector	45
pUCEF6872	pUC18 with a 5.2-kb XmaI fragment from *C. ammoniagenes* ATCC 6872	This work
pUCEFCH31	pUC18 with a 5.2-kb XmaI fragment from *C. ammoniagenes* CH31	This work
pXMJ19	Cm^r, *C. glutamicum*/*E. coli* shuttle vector	13
pXE6872	pXMJ19 containing the *nrdE*$^+$ gene from *C. ammoniagenes* ATCC 6878 (accession no. AY769914) cloned by PCR	This work
pXECH31	pXMJ19 containing the *nrdE*(Ts) gene from *C. ammoniagenes* CH31 (accession no. AY788949) cloned by PCR	This work

pair substitutions appear within a stretch of only 43 bp at nucleotide (nt) 2021 (G→A), nt 2032 (G→A), and nt 2063 (T→A). However, we consider the clustered amino acid differences at positions 674, 678, and 688, showing up only upon comparison to the NrdE sequence published in GenBank (CAA70765, gi:3077613), as irrelevant due to their absence in four other corynebacterial and five mycobacterial NrdE sequences (http://www.ncbi.nih.gov/entrez).

For recombinant expression, both the *nrdE*$^+$ and *nrdE*(Ts) genes were subcloned without any flanking regions but including their ribosome binding sites from the two sequenced plasmids, pUCEF6872 and pUCEFCH31, using the primers 5′-GGGGTCTAGATTGAAAGGCCGAGTGCTTCAAATGAC-3′ (forward) and 5′-AAAGGAGCTCTTAGAGCATGCAGGAGACGCAACC-3′ (backward). The additional XbaI site and SacI site are underlined. The start codon was changed from GTG to ATG by the forward primer. The XbaI/SacI-digested amplicons were purified prior to ligation into the XbaI/SacI-digested *E. coli*/*C. glutamicum* shuttle vector pXMJ19 (13). The fact that the resulting expression vectors, pXE6872 (*nrdE*) and pXECH31 [*nrdE*(Ts)], were first introduced into *E. coli* XL1-Blue and NrdE synthesis from the plasmid constructs was proven by immunostaining with anti-NrdE antibodies (not shown). The entire nucleotide sequence of the *nrdE*$^+$ and *nrdE*(Ts) genes was confirmed by double-strand sequencing. Then, the plasmids pXMJ19, pXE6872, and pXECH31 were introduced into the TS mutant, *C. ammoniagenes* CH31, by electroporation (40) with a BTX Electro Cell Manipulator ECM600. Only the transformant CH31/pXE6872 recovered and displayed wild-type morphology at 37°C (not shown). Expression of the introduced *nrdE*$^+$ gene and formation of fully functional holoenzyme in liquid culture (not shown) required an induction period of 4 hours prior to the temperature shift. On NBH agar (8 g nutrient broth, 2 g yeast extract, and 5 g NaCl per liter) with 1 M IPTG added, the growth defect of the TS mutant CH31 was complemented by plasmid pXE6872, whereas pXECH31 and the shuttle vector

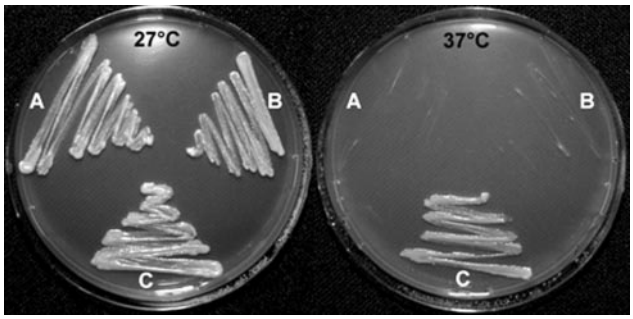

FIG. 2. Complementation of the growth defect of the TS mutant CH31 carrying the empty vector pXMJ19 (A), plasmid pXECH31 (B), or plasmid pXE6872 (C) after 3 days on NBH agar supplemented with 1 mM IPTG.

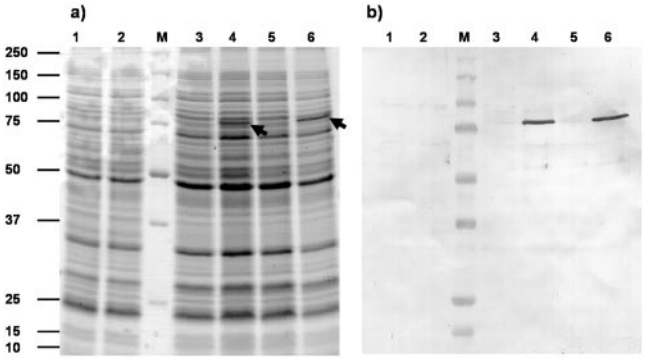

FIG. 3. Expression of *nrdE*$^+$ and *nrdE*(Ts) in *C. ammoniagenes* CH31 shown by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15) of whole-cell proteins (a) or a Western blot (41) probed with NrdE-specific antibodies (b). Molecular weight markers (Amersham Pharmacia Biotech) were run in lane M. Molecular weights (in thousands) are noted at the left. Extracts from transformants of CH31 carrying plasmids pXMJ19 are seen in lanes 1 and 2, pXE6872 in lanes 3 and 4, and pXECH31 in lanes 5 and 6. For a 4-h induction period, cells were grown in NBH broth at 27°C in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 1 mM IPTG. The arrows indicate the bands of NrdE protein which were synthesized only upon induction. Cellular proteins were obtained from 2-ml cultures of washed *C. ammoniagenes* cells after incubation in 100 μl lysis buffer (10 mM Tris-HCl, pH 6.8, 25 mM $MgCl_2$, 200 mM NaCl), containing 5 mg/ml lysozyme, for 60 min at 37°C and subsequent addition of 10 μl SDS (10%) and 100 μl loading buffer (15) prior to being heated at 95°C for 5 min.

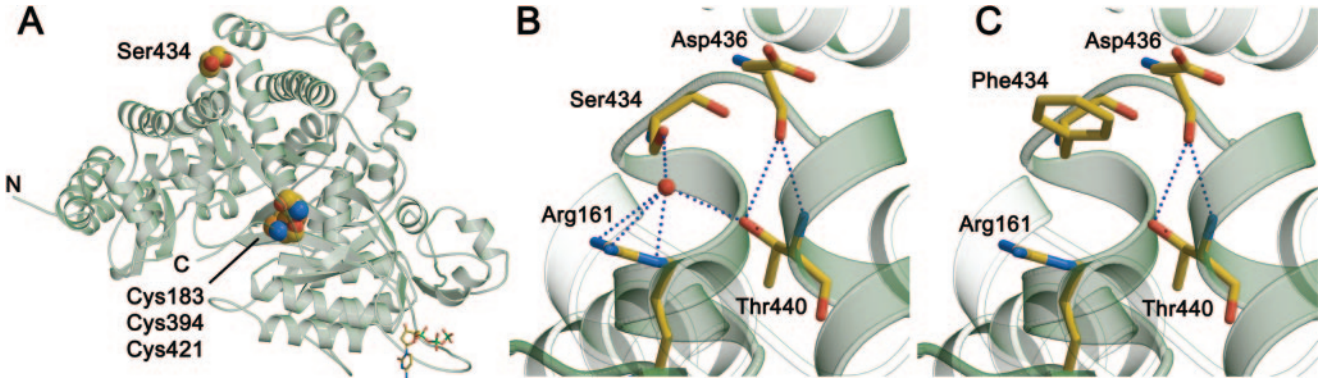

FIG. 4. Cartoon of the three-dimensional model of *C. ammoniagenes* NrdE from automated homology modeling using the "first-approach mode" of the SWISS-MODEL server (http://swissmodel.expasy.org/), with NrdE (R1E) from *Salmonella enterica* serovar Typhimurium (PDB 1PEO) as the template (12, 32, 42). The model shows the position of mutated Ser434 and the active-site cysteines as a Corey-Pauling-Koltun (CPK) model without severe gaps in the alignment (A). The model was analyzed with PROCHECK (17). Structure comparison and structural alignments were carried out with the SSAP server (26). Figures were produced using MOLSCRIPT (14) and RASTER3D (21). The position of the allosteric effector 2′-deoxycytidine-5′-triphosphate (dCTP) is modeled using the 1PEO coordinates and shown in ball-and-stick mode. The hypothetical water is shown as red spheres, and hydrogen bonds are depicted as blue dotted lines. (B) Enlarged model of the Ser434-containing surface loop showing the central water in the wild-type protein interacting via hydrogen bonds with the hydroxyl group of both Ser434 and Thr440, and with the Arg161 side-chain nitrogens. This network is further strengthened by additional hydrogen bonds from Thr440 to Asp436 to stabilize the surface-exposed loop. (C) Magnification of the same loop from the S434F mutation.

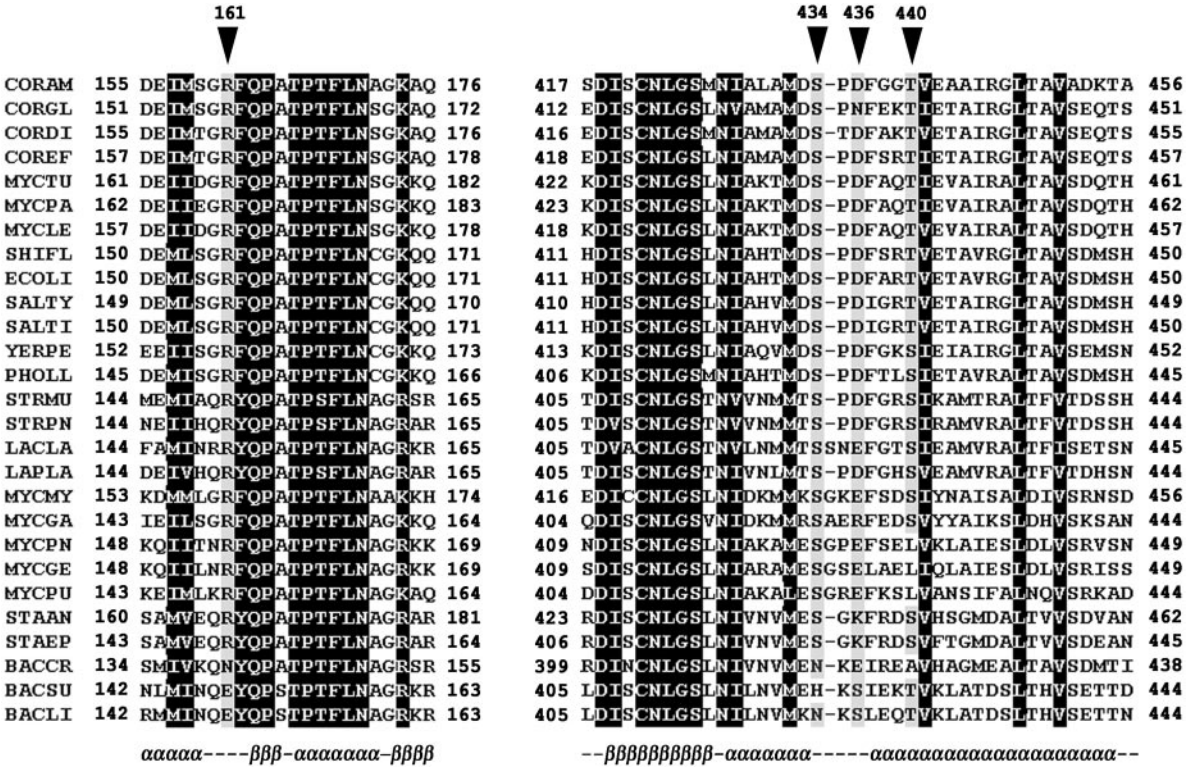

FIG. 5. Multiple alignment of NrdE proteins. The alignment was done with ClustalX (39) and shaded with GeneDoc (23) using the similarity groups of the program. Black shaded residues are 100% conserved. Ser434 and the conserved surface-exposed residues are marked with triangles on the top of the alignment and shaded yellow. Secondary structural elements are on the bottom of the alignment. Abbreviations: CORAM, *Corynebacterium ammoniagenes*; CORGL, *Corynebacterium glutamicum*; CORDI, *Corynebacterium diphtheriae*; COREF, *Corynebacterium efficiens*; MYCTU, *Mycobacterium tuberculosis*; MYCPA, *Mycobacterium paratuberculosis*; MYCLE, *Mycobacterium leprae*; SHIFL, *Shigella flexneri*; ECOLI, *Escherichia coli*; SALTY, *Salmonella enterica* serovar Typhimurium; SALTI, *Salmonella enterica* serovar Typhi; YERPE, *Yersinia pestis*; PHOLL, *Photorhabdus luminescens*; STRMU, *Streptococcus mutans*; STRPN, *Streptococcus pneumoniae*; LACLA, *Lactococcus lactis*; LAPLA, *Lactobacillus plantarum*; MYCMY, *Mycoplasma mycoides*; MYCGA, *Mycoplasma gallisepticum*; MYCPN, *Mycoplasma pneumoniae*; MYCGE, *Mycoplasma genitalium*; MYCPU, *Mycoplasma pulmonis*; STAAN, *Staphylococcus aureus*; STAEP, *Staphylococcus epidermidis*; BACCR, *Bacillus cereus*; BACSU, *Bacillus subtilis*; BACLI, *Bacillus licheniformis*.

were not effective (Fig. 2). The failure of pXECH31 was not due to inefficient expression of the introduced *nrdE*(Ts) gene since CH31/pXE6872 and CH31/pXECH31 produced the same amount of NrdE (Fig. 3) upon IPTG induction.

The finding of the nonconservative S434F exchange suggested a location for this point mutation in the tertiary structure and generated a model (720 amino acids, residues 17 to 705) of *C. ammoniagenes* NrdE (Fig. 4a) using the structure (42) of *Salmonella enterica* serovar Typhimurium NrdE (PDB code 1PEO, 68% sequence identity, r.m.s of 0.176 Å with 679 equivalent residues). Serine 434 is distant from the active (30-Å) and effector binding (60-Å) sites and located at a surface-exposed loop, connecting helix α14 with the following helix, as a very conserved residue in a structural motif, containing polar and charged residues, S434, R161, (D/E)436, and (T/S)440 common to NrdE proteins (Fig. 5). Conservation of surface hydroxyl groups in NrdE proteins may reflect a requirement for hydrogen bonding with the solvent. A hypothetical water molecule, crucial for thermostability, also present at conserved positions of other proteins (35, 36), stabilizes the surface-exposed location of Ser434 by an extensive network of hydrogen bonds (Fig. 4B). This network collapses by displacement of the central water molecule resulting from the S434F mutation, and the surface loop is destabilized (Fig. 4C). Strikingly similar properties (distance from the active site, surface exposition, lack of intramolecular hydrogen bonding) are shown by the T157I mutant, the most thermolabile variant in a series of T4 lysozyme mutants, pointing to increased thermolability resulting from increased surface hydrophobicity (3, 11, 20), while relief of reverse hydrophobic effects generates increased thermostability (28, 37, 43).

Further analysis of the S434 surface loop using the expression system developed here is suggested since neither the degradation of NrdE proteins nor leakiness of the strong *tac* promoter (this work and reference 27) occurred in *C. ammoniagenes*. Beyond this, our TS mutant CH31 may serve as a safe host strain for functional studies of foreign *nrdE* genes (e.g., from mycobacteria) restricted in the original pathogenic background, since it did not revert in the decade following its isolation.

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