Supplementary Material

Methods

Cloning of *L. monocytogenes* genes. Genomic DNA was used to amplify by PCR the *nrdA*, *nrdB*, *nrdI*, *trxL*, genes. Table S3 lists the oligonuceotide primers and restriction endonuclease used for cloning and expression of genes. DNA products were inserted into the pGEM-T Easy vector (Promega) and electroporated (BIO-RAD pulse controller plus) into *E. coli* XL1-Blue cells and transformants isolated on LB plates supplemented with 100 μg/ml of ampicillin, 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) employing standard procedures (10). Positive transformants were detected using blue-white colony screening and colony PCR. The DNA inserts were sequenced to confirm their integrity and subsequently introduced either into the appropriate expression vector (Table 1, main text).

Protein overexpression. Overnight cultures of *E. coli* BL21(λDE3) carrying the protein expression constructs were diluted to a 0.1 absorbance at 600nm in LB containing antibiotics (100 μg/ml of ampicillin for pET14b constructs and 50 μg/ml kanamycin for pET28a(+) constructs and incubated at 37°C, 250 rpm, until an absorbance of 0.6 at 600 nm was obtained. IPTG was added to a concentration of 0.1 mM and cells were further incubated for 3 h at 37°C; in the case of the *nrdA* and *nrdB* constructs incubation was at 25°C. Cultures were harvested by centrifugation at 4,000 x g for 10 min at 4°C. The supernatant was discarded and the cell pellets stored at -70°C.

Protein purification. Frozen cells were thawed and suspended in sonication buffer (50 mM Tris-HCl pH 8.5, 300 mM NaCl, 10 mM imidazole). Phenylmethylsulfonyl fluoride (PMSF) was added to the cell suspension in a final concentration of 1 mM, and the mixture was sonicated in an ultrasonic processor (Misonics) until a clear solution was obtained. The sonicate was centrifuged at 10,000 X g for 45 min at 4°C. The clear supernatant was loaded

on a HisTrap™ HP Nickel affinity column (GE Healthcare) using the AKTAprime plus system (GE Healthcare), equilibrated with the sonication buffer. The column was washed with the buffer and the proteins were eluted using a step gradient of buffer containing 50 mM, 100 mM and 250 mM imidazole. Protein samples were dialyzed against buffer containing 50 mM Tris-HCl pH 8.5, 300 mM NaCl, 5 mM dithiothreitol (DTT) and 20% glycerol. Protein recovery was monitored using the Bradford assay (2) and by SDS sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

Results

TrxL is a potential hydrogen donor for the listerial class I RNR. Studies of class I RNR enzyme activity were carried out employing NrdA, NrdB, NrdI and TrxL Ni-affinity purified His_6-tagged recombinant proteins. RNR activity was assayed by monitoring the conversion of [^{3}H]CDP to [^{3}H]dCDP (4). Conversion of CDP to dCDP was dependent on NrdA and NrdB proteins (Fig. S2 panels A and B) and on a hydrogen donor system (Fig. S2 panel C). Optimal enzyme rates were obtained in a reaction mixture containing equimolar amounts of NrdA and NrdB proteins, in 50 μl reaction mixture containing 50 mM Tris-Hcl (pH 7.6), 10mM DTT as the artificial chemical reductant, 5-10 mM ATP, [^{3}H]CDP and 5 mM $MgCl_2$ and incubated for 20 min at 30^{o}C, (Fig. S2). RNR specific activity under these conditions was 2.03 nmol of dCDP formed min^{-1} mg^{-1} protein. The reaction was 90% inhibited by the addition of 10 mM hydroxyurea (HU) (Fig. S2 panel F), which is a potent inhibitor of the oxygen dependent class I RNR radical machinery (3, 9). Many class Ia RNRs (but not class Ib RNRs) contain a segment of ~100 amino acids in the N-terminal portion of the NrdA subunit termed the ATP-cone domain (1). The ATP-cone domain functions to bind ATP or dATP to allosterically regulate overall enzyme activity (8). We identified an amino acid sequence present in the N-terminal portion of the *L. monocytogenes* NrdA (and in other listerial NrdA proteins) with

significant similarity to both the PROSITE and InterPro ATP-cone profiles (PS51161 and IPR005144, respectively). To determine whether the activity of the *L. monocytogenes* class I RNR is modulated by ATP and dATP, assays were carried out in the presence of 0-15 mM ATP and 0-10 mM dATP. Maximum activity was obtained with 3 mM ATP (Fig. S2 panel D), stimulation was about 60 fold above that found in the absence of ATP. In contrast, dATP alone had no effect on activity. When 1 mM dATP was added to a reaction mixture containing 5 mM ATP, enzyme activity was inhibited by 90% (Fig. S2 panel E). These results conform to the typical pattern observed for class Ia RNRs and place the *L. monocytogenes* aerobic RNR in the class Ia subdivision (5). To assess whether TrxL, the thioredoxin-like protein encoded in the class Ia RNR operon, can serve as an electron donor for the NrdAB reaction we performed assays in the presence of 1-2 mM DTT and variable amounts of TrxL protein. TrxL significantly stimulated RNR activity (Fig. S3). These results support the view that TrxL, together with thioredoxin reductase and NADPH, may function *in vivo* as a potential electron donor for the aerobic class I RNR as has been previously described for the NrdH proteins of *E. coli* and *Staphylococcus aureus* (6, 7).

References to Methods and Results

1. **Aravind, L., Y. I. Wolf, and E. V. Koonin.** 2000. The ATP-cone: an evolutionarily mobile, ATP-binding regulatory domain. J. Mol. Microbiol. Biotechnol **2:**191-194.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.
3. **Ehrenberg, A., and P. Reichard.** 1972. Electron spin resonance of the iron-containing protein B2 from ribonucleotide reductase. J. Biol. Chem. **247:**3485-3488.

4. **Engstrom, Y., S. Eriksson, L. Thelander, and M. Akerman.** 1979. Ribonucleotide reductase from calf thymus. Purification and properties. Biochemistry **18:**2941-2948.

5. **Jordan, A., and P. Reichard.** 1998. Ribonucleotide reductases. Annu. Rev. Biochem. **67:**71-98.

6. **Masalha, M., I. Borovok, R. Schreiber, Y. Aharonowitz, and G. Cohen.** 2001. Analysis of transcription of the *Staphylococcus aureus* aerobic class Ib and anaerobic class III ribonucleotide reductase genes in response to oxygen. J. Bacteriol. **183:**7260-7272.

7. **Rabinovitch, I., M. Yanku, A. Yeheskel, G. Cohen, I. Borovok, and Y. Aharonowitz.** 2010. *Staphylococcus aureus* NrdH redoxin is a reductant of the class Ib ribonucleotide reductase. J. Bacteriol. **192:**4963-4972.

8. **Reichard, P.** 2002. Ribonucleotide reductases: the evolution of allosteric regulation. Arch Biochem. Biophys. **397:**149-155.

9. **Reichard, P., and A. Ehrenberg.** 1983. Ribonucleotide reductase--a radical enzyme. Science **221:**514-519.

10. **Sambrook, J., Fritsch, E. F., T. Maniatis.** 1989. Molecular Cloning: a laboratory manual, vol. Cold Spring Harbor, NY.

Legends to Figures

Figure S1. The *L. monocytogenes* EGD-e class I RNR *nrdABI-trxL* gene cluster form an operon. Co-transcription of the *nrdA-nrdB, nrdB-nrdI*, *nrdI-trxL* and *trxL-mazG* genes was tested under aerobic and anaerobic growth conditions. The *rpoD* gene, which encodes a RNA polymerase sigma subunit, served as a positive control. Transcription of the *mazG* gene was assayed separately (data not shown). For each gene/gene pair three RT reactions were

performed, with reverse transcriptase (RT) (+), without RT (-), and a genomic DNA control (C). Standard PCR reactions were run employing the primers shown in Table S1. Oligonucleotide primers used in RT-PCR to demonstrate co-transcription of genes, and in 5'-RACE experiments to determine nrdA and nrdD transcription start sites, are shown in Table S1**.** The *nrdABI-trxL* genes are transcribed as a single operon under both aerobic and anaerobic conditions. Although a Rho-dependent termination site is present down-stream of the *trxL* gene, the *mazG* gene was also found to be co- transcribed with the class I RNR operon in agreement with Northern blot analysis that revealed a transcript corresponding in size to that for the *nrdA-mazG* genes (data not shown).

Figure S2. ATP and dATP regulate the activity of the *L. monocytogenes* EGD-e class I RNR. RNR activity was assayed by monitoring the conversion of [^{3}H]CDP to [^{3}H]dCDP (see above). Panels: A. Constant NrdB concentration (23μg per 50 μl reaction mixture) with increasing NrdA concentration; B. Constant NrdA concentration (48μg per 50 μl reaction mixture) with increasing NrdB concentration; C. Increasing DTTconcentrations; D. Increasing ATP concentration; E. Increasing dATP concentration in the presence of the optimal ATP concentration; F. Effect of hydoxyurea on activity.

Figure S3. TrxL stimulates the L. monocytogenes EGD-e class I NrdAB RNR activity. Reactions were carried out with NrdA (36 μg) and NrdB (24 μg) incubated in the standard assay mixture (see above) plus 0-10 mM DTT. TrxL (24 μg) was added with 2 mM DTT.

Figure S4. Molecular phylogeny of bacterial class I RNR NrdA and NrdE proteins. The neighbor-joining (NJ) method was applied to estimate relationships among aligned sequences by using the MEGA 4 program. *Archaeoglobus fulgidus* DSM 4304 class II RNR NrdJ amino

acid sequence (accession number AAB89584) was used as the outgroup in the tree. Bootstrap analysis using NJ was conducted with 500 replicates, and values above 50% are given at nodes. Bacterial names and accession numbers of NrdA and NrdE proteins are listed in Table S5.

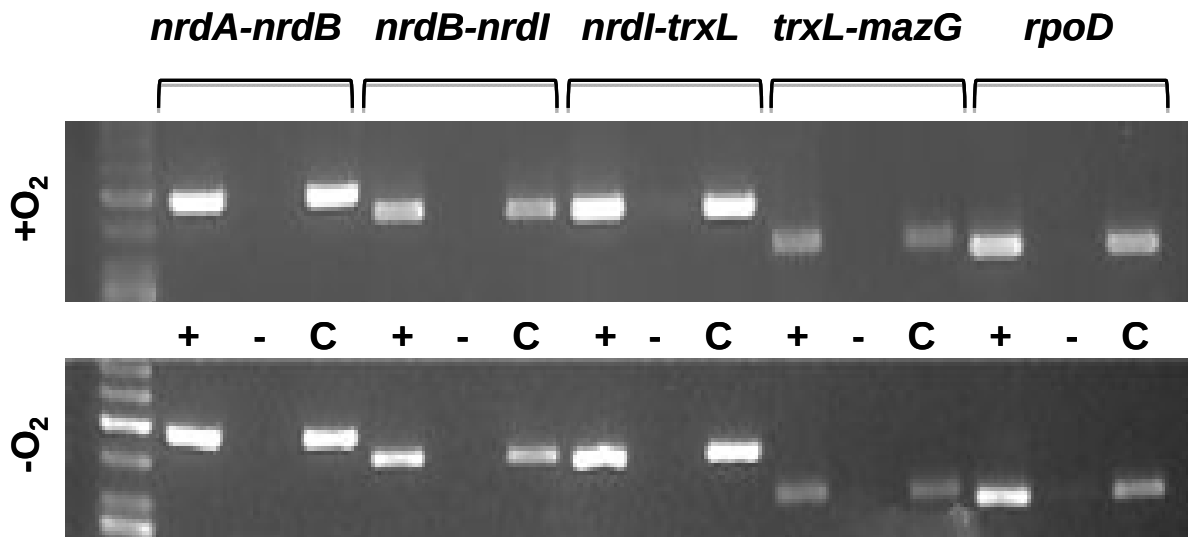

Figure S1

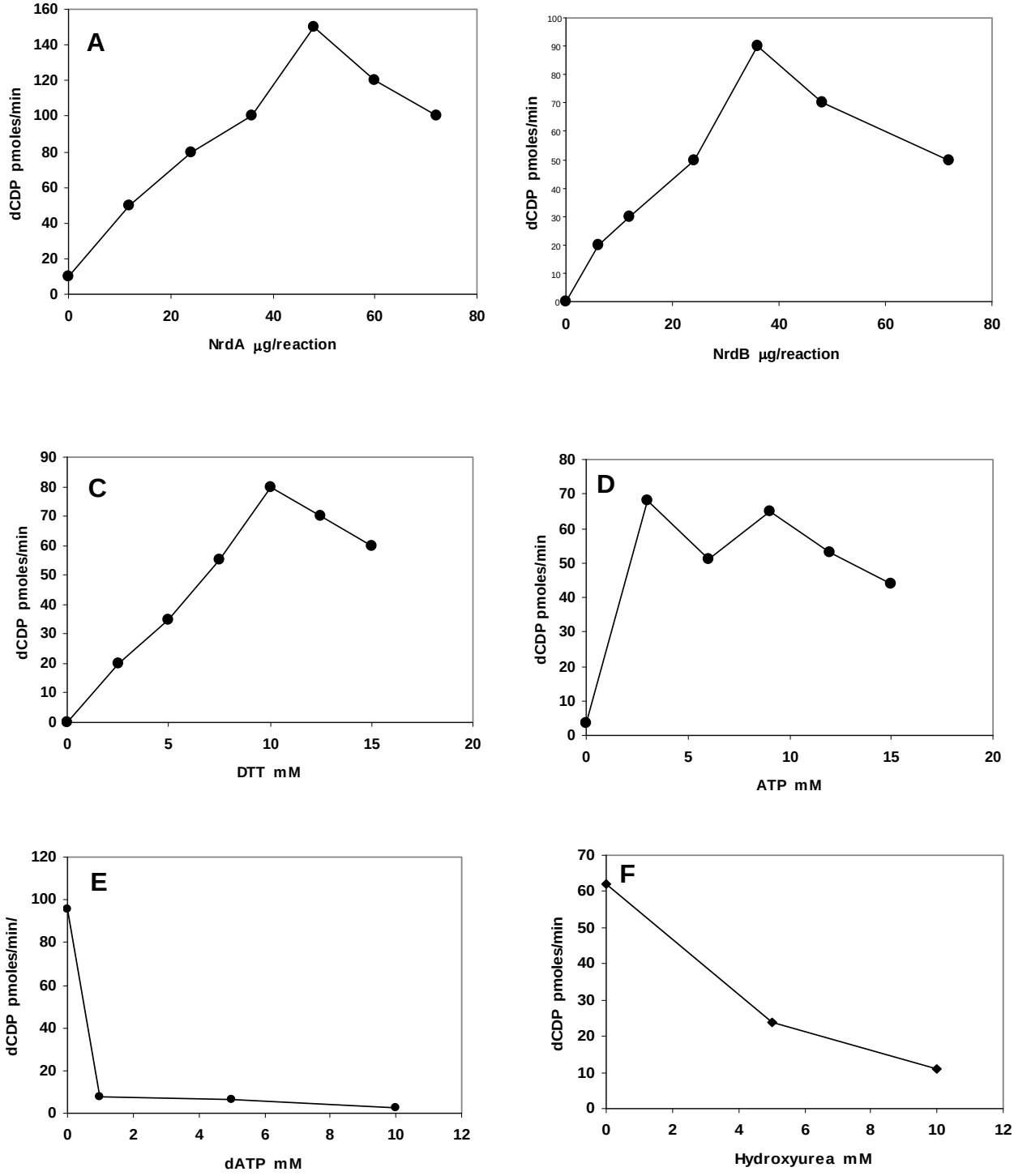
A
dCDP pmoles/min
NrdA µg/reaction
B
dCDP pmoles/min
NrdB µg/reaction
C
dCDP pmoles/min
DTT mM
D
dCDP pmoles/min
ATP mM
E
dCDP pmoles/min/
dATP mM
F
dCDP pmoles/min
Hydroxyurea mM

Figure S2

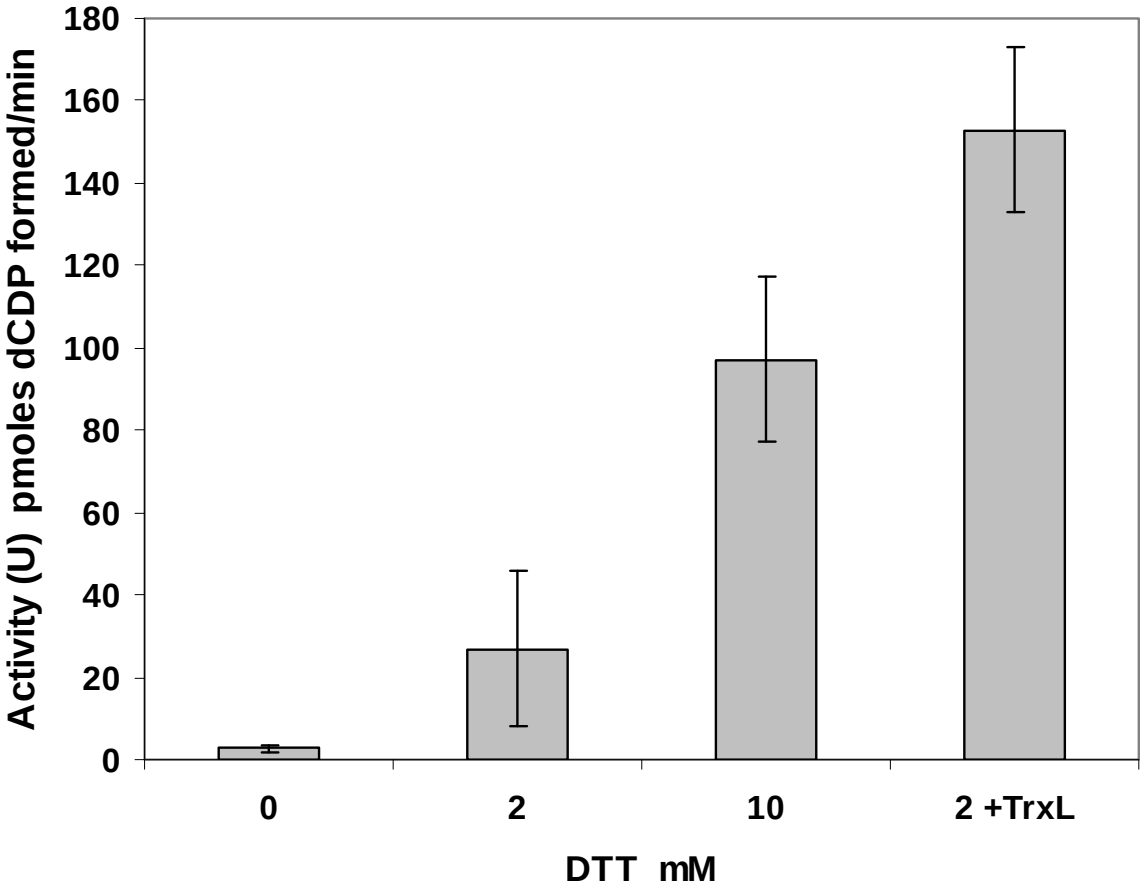
Activity (U) pmoles dCDP formed/min
180
160
140
120
100
80
60
40
20
0
0
2
10
2 +TrxL
DTT mM

Figure S3

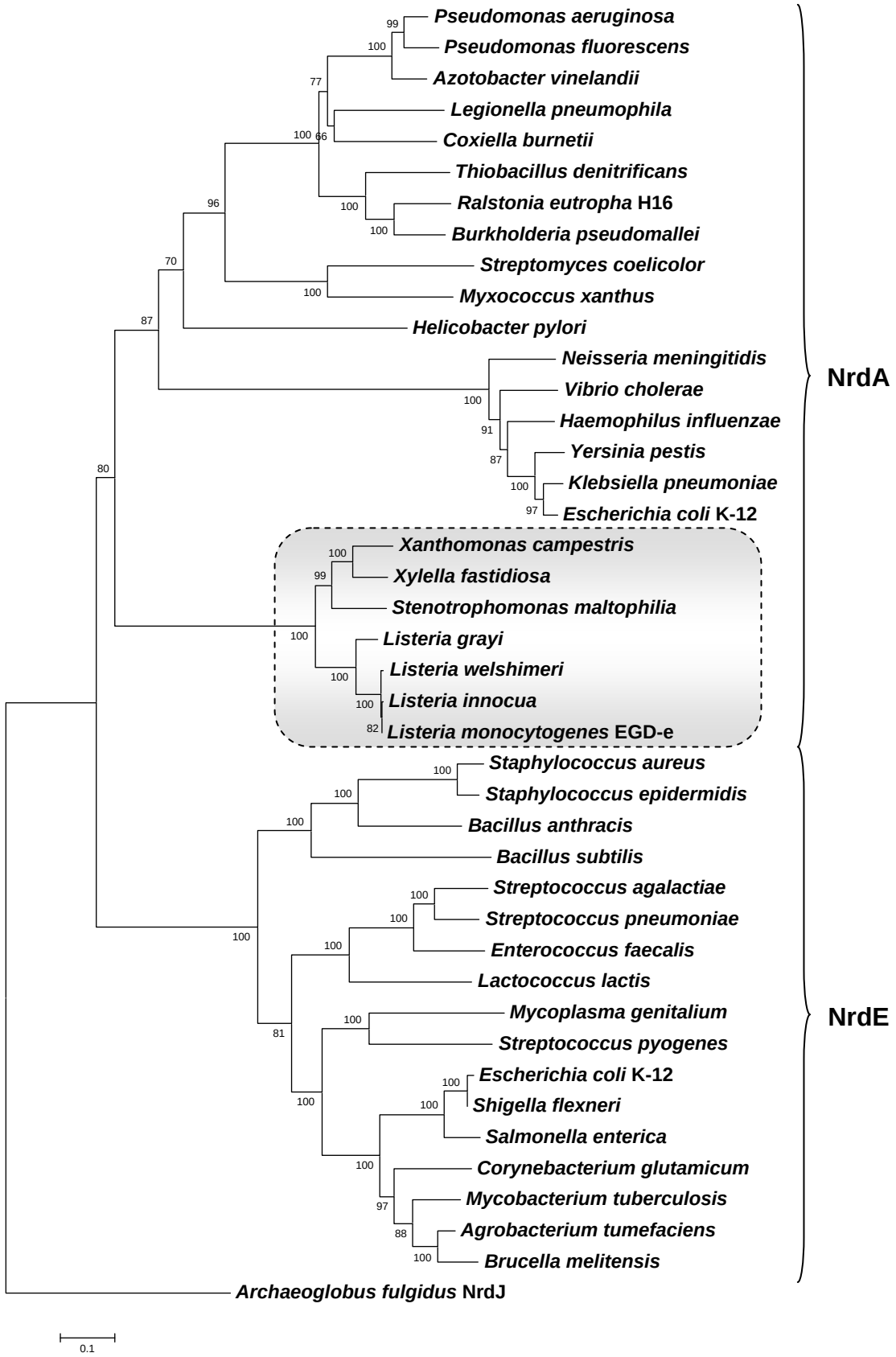
Pseudomonas aeruginosa
Pseudomonas fluorescens
Azotobacter vinelandii
Legionella pneumophila
Coxiella burnetii
Thiobacillus denitrificans
Ralstonia eutropha H16
Burkholderia pseudomallei
Streptomyces coelicolor
Myxococcus xanthus
Helicobacter pylori
Neisseria meningitidis
Vibrio cholerae
Haemophilus influenzae
Yersinia pestis
Klebsiella pneumoniae
Escherichia coli K-12
Xanthomonas campestris
Xylella fastidiosa
Stenotrophomonas maltophilia
Listeria grayi
Listeria welshimeri
Listeria innocua
Listeria monocytogenes EGD-e
Staphylococcus aureus
Staphylococcus epidermidis
Bacillus anthracis
Bacillus subtilis
Streptococcus agalactiae
Streptococcus pneumoniae
Enterococcus faecalis
Lactococcus lactis
Mycoplasma genitalium
Streptococcus pyogenes
Escherichia coli K-12
Shigella flexneri
Salmonella enterica
Corynebacterium glutamicum
Mycobacterium tuberculosis
Agrobacterium tumefaciens
Brucella melitensis
Archaeoglobus fulgidus NrdJ
NrdA
NrdE
0.1

Figure S4

Tables

Table S1. **Oligonucleotide primers used in RT-PCR and 5'-RACE experiments**.

Primer name	Primer sequence (5'➔3')	Comments
RT-PCR primers		
nrdA1F	CGCGTTACTTAGCGACTGAC	
nrdA1R	ACGGATAGCAGCTCCTGTTG	
nrdA2F	GTGCCAAGTGGTATCAAAGC	
nrdB1F	AGCCTATGCAGATAACTTTACAG	
nrdB1R	TGTTTGCGGAGAATCCAGTG	
nrdB2F	CCCGTATCCTTCTTTCTACCG	
nrdB2R	GCGTCCATGATTGGCTCATTAC	
nrdI1F	AGCGAAGGGCATGAAGTTG	
nrdI1R	TTTGCCATTCGGTCGACAG	
nrdI2F	TTGCGGAGCTGTCGACCGAATG	
nrdI2R	TTTGCCATTCGGTCGACAG	
trx1R	GTGGATCTCTTCGCCGCCTTTG	
trx1F	TTTACAAAGGCGGCGAAGAG	
trx2F	GCGCTAAGGAGTGGAATCAAATGAC	
trx2R	GGATCTCTTCGCCGCCTTTG	
mazG1F	AGAGCGTGATTGGCTAGATC	
mazG1R	TCTGCTGCTTCAAGTGAC	
mazG2R	TTTGGCCAAGTTCTCCTACG	
rpoD1F	CTGCCAGATTAGCTCCATTC	
rpoD1R	TTCAATGTCGCCAGCTTC	
5'-RACE primers		
nrdA_5RACE_FOR1	GAACGACCACAAATAGTTC	
nrdA_5RACE_REV1	ATATCTGTACGAGCTTCTG	
nrdA_5RACE_REV2	CCGCGTAATCTTCCTTCTTCTC	
nrdA_5RACE_REV3	TCGGCTTTTGTCAAAAGGTAG	
nrdD_5RACE_FOR	TTCGTAATGCCGTTGAAGC	
nrdD_5RACE_REV1	CAGTCGCGTCTTTGTTGG	
nrdD_5RACE_REV2	GCGGTACTCTATGTAAGCC	
nrdD_5RACE_REV3a	TCTGCCTTGTTAGGTAATTCAC	

Table S2. ***Listeria monocytogenes*** **genomes analyzed in this study.**

serovar	strain[1]	NrdD[2]	genome accession number and status[3]
1/2a	EGD-e (ATCC BAA-679)	6-aa deletion	NC_003210; complete
	SLCC 5764 (“Mackaness”)	WT	this study
	08-5578	WT	NC_013766; complete
	08-5923	WT	NC_013768; complete
	10403S (DP-L184)	WT	AARZ02000008; incomplete
	F6854	WT	AADQ01000021; incomplete
	F6900	WT	AARU02000005; incomplete
	FSL J2-003	WT	AARM02000013; incomplete
	FSL N3-165	WT	AARQ02000010; incomplete
	J0161 (FSL R2-499)	WT	AARW02000006; incomplete
	J2818	WT	AARX02000005; incomplete
1/2b	FSL J1-194	WT	AARJ02000007; incomplete
	FSL J1-175	WT	AARK02000110; incomplete
	FSL R2-503 (G6054)	WT	AARR02000009; incomplete
1/2c	FSL R2-561	WT	AARS01000004; incomplete
3a	Finland 1988	WT	AART01000004; incomplete
4a	HCC23	WT	NC_011660; complete
	L99	WT	FM211688; complete
	FSL F2-208	WT	ADXE01000424; incomplete
4b	Clip81459 (CLIP 80459)	WT	NC_012488; complete
	F2365	WT	NC_002973; complete
	FSL N1-017	WT	AARP04000007; incomplete
	H7858	WT	AADR01000001; incomplete
	HPB2262 (Aureli 1997)	WT	AATL02000009; incomplete
4c	FSL J2-071	WT	AARN04000005; incomplete

[1] strain L99 is reported as serovar 4a in the submission but is reported as serovar 4c in the literature (see for example Moorhead and Dykes. 2003. Curr Microbiol).

[2] “WT” encodes an intact NrdD; “6-aa deletion” refers to the NrdD KITPFE deletion described in this study (see text).

[3] accession numbers and genome status are provided according to GenBank data.

Table S3. **Oligonucleotide primers used for construction of recombinant protein overexpression plasmids**.

RT-PCR primers		
Primer name	**Primer sequence (5'➔3')**	**Notes***
LMO_nrdA_NdeI_FOR1	CAGTCATATGAAATGGGGATTGCAAATG	NdeI
LMO_nrdA_Bpu1102I_REV1	ATGCTCAGCTTGAGCACCATTCG	Bpu1102I
LMO_nrdB_XhoI_FOR1	ACGCTCGAGATGGCTAACCAAAAAG	XhoI
LMO_nrdB_Bpu1102I_REV1	GCGCGCTCAGCTCATAAATCGTCG	Bpu1102I
LMO_nrdI_NdeI_FOR1	GCGCATATGAGAATCTTGTTAGCC	NdeI
LMO_nrdI_Bpu1102I_REV1	GCGGCTCAGCTCATTTGATTCCAC	Bpu1102I
LMO_trxL_NdeI_FOR2	GCACGGTCATATGACAAGTATTGAAATTAAATCACC	NdeI
LMO_trxL_Bpu1102I_REV2	ATAGCTCAGCTTACGCGTTTAATGAAACTGC	Bpu1102I

* Names of restriction enzymes whose recognition sites are underlined in sequences of corresponding primers.

Table S4. **Oligonucleotide primers used for construction of deletion and insertion *nrdD* mutants in *L. monocytogenes* strains**.

Primer name	Primer sequence (5'➔3')*	Comments
Δ*nrdDG* in *L. monocytogenes* strain EGD-e		
LMOnrdD_A_SacI	ATTAGAGCTCAGGGCTTACATAGAGTACC	Complimentary nucleotides are shown in bold
LMOnrdD_B	**CTTTGTCGCCTTCCCATTC**ACCGCCGTAAAA TACAGTTGC	
LMOnrdG_C	**GAATGGGAAGGCGACAAAG**TGGACAGGCTAC ACTTGG	
LMOnrdG_D_XbaI	TAATTCTAGAGGCTGCTAATAATGGATGC	
nrdDG_ADflank_FOR	GGACAAAGAGCGATTCTG	
nrdDG_ADflank_REV	CAATACGCACCCAATAGC	
18 nucleotides deletion encoding KITPFE amino acids in *nrdD* of *L. monocytogenes* strain F2365		
4bF2365_nrdD_A_SacI	ATTAGAGCTCGCCCGAAATCTATCCAAACTG C	Complimentary nucleotides are shown in bold Identical in both F2365 and EGD-e genomes Primers that were used to confirm EGD-e and F2365 *ndrD* mutants
4bF2365_nrdD_B	**TTTACGAACATCATAGTGGAAAGAG**	
4bF2365_nrdD_C	**CTCTTTCCACTATGATGTTCGTAAA**AAAATT GATTTTGAAAAAGATTATCC	
4bF2365_nrdD_D_XbaI	TAATTCTAGACTAGTACTATCGCTGCTTATA GCAATC	
nrdD_EGDeCOMPfnkF	GGAGCATTTGGCAAACGACTAC	
nrdD_EGDeCOMPfnkR	CCACAGCTTGGGCATT**t**AAAGCCTTC	
Insertion of 18 nucleotides encoding KITPFE amino acids in *nrdD* of *L. monocytogenes* strain EGD-e		
EGDe_nrdD_A_SacI	ATTAGAGCTCAGCCCATACCATGCAATGACC	Complimentary nucleotides are shown in bold
EGDe_nrdD_B	CAATTTT**TTCAAATGGGGTAATTTT**TTTACG AACATCATAGTGGAAAGAG	
EGDe_nrdD_C	GTAAA**AAAATTACCCCATTTGAA**AAAATTGA TTTTGAAAAAGATTATCC	
EGDe_nrdD_D_XbaI	TAATTCTAGACTAGTACTATCGCTGCTTATA GCAATC	
pKSV7 multiple cloning site flanking primers		
pKSV7_MCSflank_FOR	TTCACGTGTTCGCTCATGG	
pKSV7_MCSflank_REV	GCGGGCCTCTTCGCTATTAC	

* Nucleotides of restriction sites are underlined.

Table S5. Bacterial names and accession numbers of corresponding NrdD, NrdA and NrdE proteins that were used for phylogeny analyses (Figure 6, main text [NrdD]; Figure S4, Supplementary Material [NrdA and NrdE]).

Organism name	**NrdD accession number**	**NrdA accession number**	**NrdE accession number**
Enterobacteria phage T4	AAD42633		
Actinobacillus succinogenes 130Z	ABR74766		
Agrobacterium tumefaciens C58			AAK85889
Azotobacter vinelandii DJ		ACO79483*	
Bacillus anthracis Ames			AAP25314
Bacillus subtilis 168			CAA92810
Brucella melitensis biovar Abortus 2308			CAJ13055
Burkholderia pseudomallei 1106a		ABN91742	
Chromobacterium violaceum ATCC 12472	AAQ60084		
Citrobacter koseri ATCC BAA-895	ABV14659		
Corynebacterium glutamicum ATCC 13032			CAF21192
Coxiella burnetii RSA 331		ABX79118	
Enterococcus faecalis V583	AAO82452		AAO80326
Escherichia coli K12 MG1655	AAC77195	AAC75294	AAC75722
Haemophilus influenzae Rd KW20		AAC23305	
Helicobacter pylori J99		AAD06201	
Klebsiella pneumoniae 342	ACI10318	ACI07284	
Lactobacillus casei ATCC 334	ABJ68995		
Lactococcus lactis Il1403	AAK04371		AAK05073
Legionella pneumophila str. Paris		CAH12890	
Listeria grayi DSM 20601	EFI83996	EFI82948	
Listeria innocua Clip11262	CAC95538	CAC97487	
Listeria monocytogenes 4b F2365	AAT03086		
Listeria monocytogenes EGD-e	CAD00806	CAD00233	
Listeria welshimeri SLCC5334	CAK19671	CAK21590	
Mannheimia succiniciproducens MBEL55E	AAU37240		
Mycobacterium tuberculosis H37Rv			CAA16136
Mycoplasma genitalium G37			AAC71452
Myxococcus xanthus DK 1622		ABF86509	
Neisseria meningitides MC58		AAF41667	
Pasteurella multocida Pm70	AAK03024		
Photobacterium profundum SS9	CAG22315		
Photorhabdus luminescens subsp. *laumondii* TTO1	CAE16871		
Pseudomonas aeruginosa PAO1		AAG04545	
Pseudomonas fluorescens Pf0-1		ABA75983	
Psychromonas ingrahamii 37	ABM02592		
Pyrococcus furiosus DSM 3638	AAL82095		
Ralstonia eutropha H16		CAJ94310	

Salmonella enterica serovar Typhi			CAD05918
Serratia proteamaculans 568	ABV39633		
Shewanella baltica OS155	ABN61177		
Shigella dysenteriae Sd197	ABB64158		
Shigella flexneri 2a str. 301			AAN44196
Staphylococcus aureus COL	AAW38633		AAW37848
Staphylococcus epidermidis ATCC 12228	AAO05814		AAO04110
Stenotrophomonas maltophilia K279a		CAQ46303	
Streptococcus agalactiae 2603V/R	AAN00945		AAM99706
Streptococcus gordonii Challis substr. CH1	ABV10652		
Streptococcus mutans UA159	AAN59670		
Streptococcus pneumoniae R6			AAK99869
Streptococcus pyogenes M1 GAS			AAK33449
Streptococcus suis 98HAH33	ABP93282		
Streptococcus thermophilus LMG 18311	AAV61557		
Streptomyces coelicolor A3(2)		CAB82485	
Thiobacillus denitrificans ATCC 25259		AAZ97929	
Vibrio cholerae O1 biovar El Tor str. N16961		AAF94415	
Vibrio fischeri ES114	AAW87351		
Xanthomonas campestris ATCC 33913		AAM43207	
Xylella fastidiosa 9a5c		AAF84006	
Yersinia pestis KIM 10	AAM84321	AAM86525	

* The original deduced amino acid sequence contains an intein that was removed from the sequence before using in alignments.