

Research article

Structural, functional, and evolutionary analysis of *moeZ*, a gene encoding an enzyme required for the synthesis of the *Pseudomonas* metabolite, pyridine-2,6-bis(thiocarboxylic acid)

Marc S Cortese¹, Allan B Caplan² and Ronald L Crawford*¹

Address: ¹Environmental Research Institute, University of Idaho, Moscow, ID 83844-1052, USA and ²Microbiology Molecular Biology & Biochemistry Department, University of Idaho, Moscow, ID 83844-2339, USA

E-mail: Marc S Cortese - mcortese@uidaho.edu; Allan B Caplan - acaplan@uidaho.edu; Ronald L Crawford* - crawford@uidaho.edu

*Corresponding author

Published: 23 April 2002

Received: 20 November 2001

BMC Evolutionary Biology 2002, 2:8

Accepted: 23 April 2002

This article is available from: <http://www.biomedcentral.com/1471-2148/2/8>

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Abstract

Background: Pyridine-2,6-bis(thiocarboxylic acid) (pdtc) is a small secreted metabolite that has a high affinity for transition metals, increases iron uptake efficiency by 20% in *Pseudomonas stutzeri*, has the ability to reduce both soluble and mineral forms of iron, and has antimicrobial activity towards several species of bacteria. Six GenBank sequences code for proteins similar in structure to MoeZ, a *P. stutzeri* protein necessary for the synthesis of pdtc.

Results: Analysis of sequences similar to *P. stutzeri* MoeZ revealed that it is a member of a superfamily consisting of related but structurally distinct proteins that are members of pathways involved in the transfer of sulfur-containing moieties to metabolites. Members of this family of enzymes are referred to here as MoeB, MoeBR, MoeZ, and MoeZdR. MoeB, the molybdopterin synthase activating enzyme in the molybdopterin cofactor biosynthesis pathway, is the most characterized protein from this family. Remarkably, lengths of greater than 73% nucleic acid homology ranging from 35 to 486 bp exist between *Pseudomonas stutzeri moeZ* and genomic sequences found in some *Mycobacterium*, *Mesorhizobium*, *Pseudomonas*, *Streptomyces*, and cyanobacteria species.

Conclusions: The phylogenetic relationship among *moeZ* sequences suggests that *P. stutzeri* may have acquired *moeZ* through lateral gene transfer from a donor more closely related to mycobacteria and cyanobacteria than to proteobacteria. The importance of this relationship lies in the fact that pdtc, the product of the *P. stutzeri* pathway that includes *moeZ*, has an impressive set of capabilities, some of which could make it a potent pathogenicity factor.

Background

Pyridine-2,6-bis(thiocarboxylic acid) (pdtc) is an iron-regulated metabolite produced by certain pseudomonads that is highly reactive towards metals by virtue of a pair of thiocarboxylate groups and ring nitrogen that combine to form a non-specific, high affinity, tri-dentate ligand (Fig-

ure 1)[1,2]. The formation constants for Co, Cu, and Fe complexes of pdtc were determined to be greater than 10^{33} [3]. Uptake studies using ⁵⁹Fe showed that addition of pdtc to the growth medium of two different pdtc-producing pseudomonads increased the efficiency of iron uptake by 20% [1]. The contribution of pdtc to iron uptake

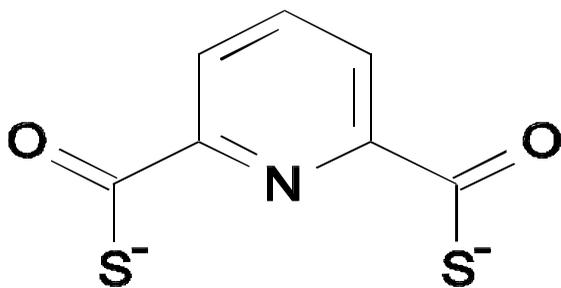


Figure 1
Structure of pdtc.

may be due to the fact that the copper complex of pdtc (Cu:pdtc) has the ability to reduce both soluble and mineral forms of ferric iron [1]. Not surprisingly, in light of its high affinity for essential metals, pdtc also exhibits antimicrobial activity [4].

MoeB, the molybdopterin synthase activating enzyme (MSAE), is part of the well-characterized molybdopterin cofactor (MPT-Mo) synthesis pathway. A dimer of MoeB adenylates the MoeD subunits of molybdopterin synthase that, after the conversion of these AMP moieties to thiocarboxylates by a sulfurtransferase, goes on to convert precursor Z to molybdopterin (MPT) [5,6]. Molybdopterin synthase is a heterotetrameric protein composed of two units each of MoeD and MoeE. The crystal structure of the *E. coli* MoeB-MoeD complex clearly shows the interaction between these two proteins and unambiguously confirms the adenylation role of MoeB [7]. After a thiocarboxylate moiety is formed on the terminal glycine of each MoeD subunit, the two sulfur atoms are transferred to precursor Z as sulfhydryl groups in a dithiolene configuration. [8–11]. Molybdenum is added to MPT in subsequent steps to generate MPT-Mo, the active form of the molybdopterin cofactor. Since MPT-Mo is required for nitrate reductase activity [12], the activity of this enzyme can be used as a diagnostic indicator for the presence of the MPT-Mo synthetic pathway [7].

There are a number of genomic sequences deposited in GenBank as MoeB proteins, although only a few have been physiologically or genetically proven to function in MPT-Mo synthesis. Two *moeB* sequences with evidence for function include a knockout of the open reading frame encoding GenBank accession number BAA35521, which was shown to cause the accumulation of precursor Z in *E. coli* [13] and accession number Z95150, which was found to be located within a cluster of other MPT-Mo synthesis genes of *Mycobacterium tuberculosis* [14]. MSAEs from eukaryotic organisms have also been characterized: MOCS3

(GenBank accession number NM_014484) from humans and *cnx5* from *Arabidopsis thaliana* [15], respectively, have been sequenced, and *cnxF* from *Aspergillus nidulans* [16] has been both sequenced and physiologically characterized.

MoeZ is one of several MSAE-like conceptually translated protein sequences identified by the *M. tuberculosis* genome sequencing group at Sanger Center [14]. Although coding for a putative protein with high sequence similarity to MoeB, *moeZ* was so named because it had no genetic linkage to other MPT-Mo synthesis genes. Cole *et al.* [14] state in their GenBank entry (accession number Z95120) that MoeZ is possibly involved in the synthesis of molybdopterin, but there has been no biochemical or physiological characterization of this protein. An open reading frame (ORF-F) identified as a necessary part of the *Pseudomonas stutzeri* *pdt* locus coding for the synthesis pathway of pdtc (Figure 1) was found to have 57% identity to *M. tuberculosis* MoeZ at the amino acid (AA) level. Further analysis revealed that a 521 base pair region of *P. stutzeri* ORF-F shares 80% homology with *M. tuberculosis* *moeZ* [17]. The products of the *pdt* locus probably function by activating, then sulfurating, a carboxylic acid precursor. The end product of this synthesis is pdtc, a molecule with two thiocarboxylic acid groups that is secreted from the cell.

Recently, data obtained from genome sequencing projects have made it clear that bacteria exchange genetic information by means of transducing bacteriophages, conjugative plasmids, and natural transformation [18] at a much higher rate than previously thought [19]. There seem to be few insurmountable barriers to interspecies gene transfer, with transfers being documented between such diverse organisms as archae and eubacteria [20]. *Synechococcus* sp. [21] and some *P. stutzeri* strains [22] are two examples of the many bacterial species known to undergo natural transformation. Of the completely sequenced genomes, all show some level of lateral gene transfer (LGT). For example, 3.3% and 16.6% of the *M. tuberculosis* and *Synechocystis* sp. PCC6803 genomes, respectively, are estimated to be composed of sequences acquired by LGT [19].

We report here on the structural, functional, and evolutionary relationships of *moeZ* and discuss the possibility that the discontinuous distribution of *moeZ* is the result of a LGT event between unrelated bacteria.

Results and Discussion

Structure

We assembled a set of sequences similar to the product of *Pseudomonas stutzeri* *pdt* locus ORF-F (*Ps-moeZ*) (Table 1). Protein BLAST searches using *Ps-moeZ* as the query yielded more than 100 sequences with significant similarity

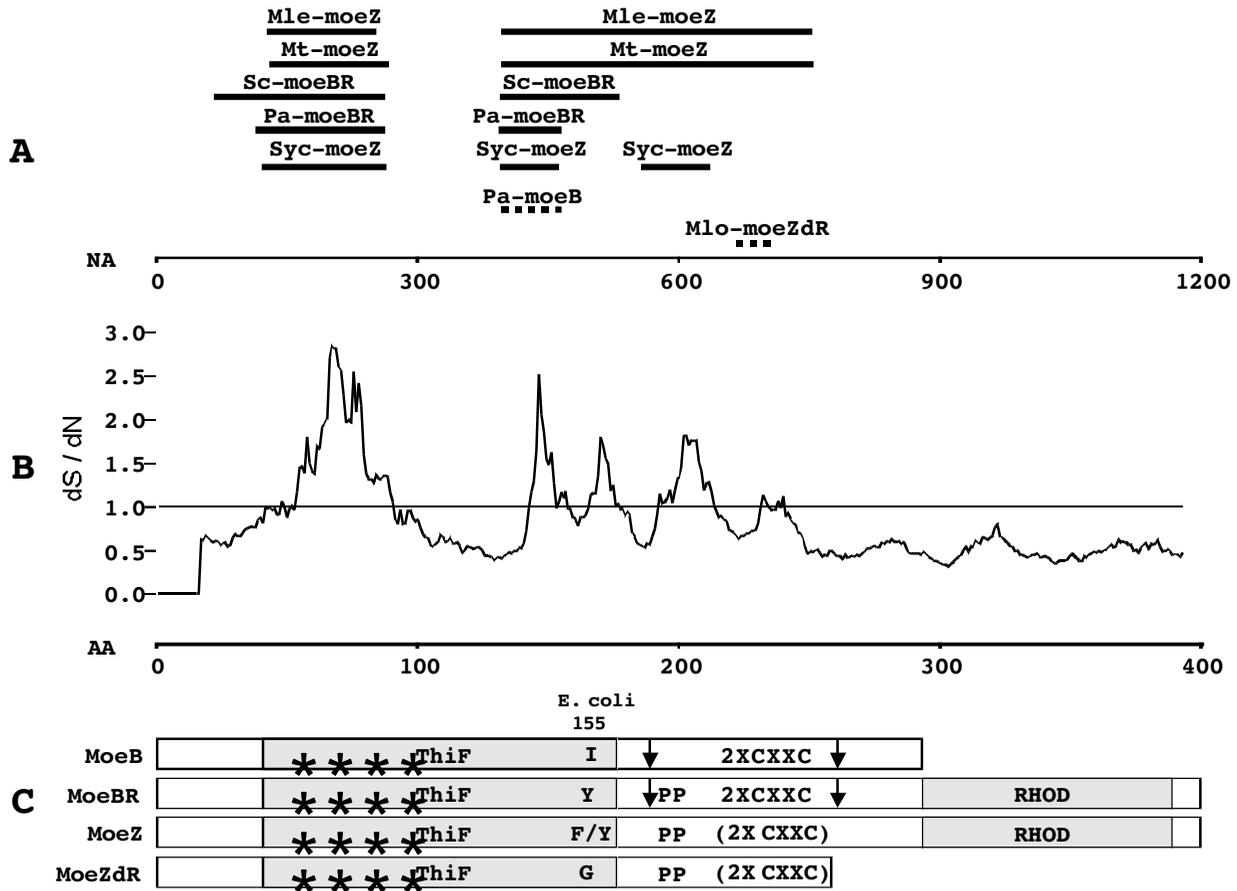


Figure 2

Domain and homology map of MoeB/BR/Z sequences. **A.** Regions of NA homology between *Ps-moeZ* and other sequences in this study. Homologous regions between the members of the high-homology group and *Ps-moeZ* are depicted as solid lines. Regions of *Ps-moeZ* homology to other strains are shown with dotted lines. The horizontal scale is based on the NA alignments with 0 being the first base of *Mle-moeZ*. **B.** Moving average of the ratio of the synonymous to non-synonymous nucleotide substitutions (dS/dN) per codon found among the high homology group using an 18 bp window. Scale is based on the AA alignment with 0 being the first residue of *Mle-moeZ*. **C.** Structure of MoeB/BR/Z conceptual proteins. The bars labeled with protein designations indicate the length of each structural class and the inclusion or omission of domains and motifs. ThiF = ThiF family domain; 2X CXXC = MoeB C-terminal domain containing tandem cysteine pairs, MoeBR central domain containing tandem cysteine pairs, or MoeZ central domain with modified regions in place of cysteine pairs; RHOD = Rhodanese-like domain. Locations of CXXC motifs are indicated by vertical arrows. **** = dinucleotide binding motif. (2X CXXC) = modified 2X CXXC domain. Location of *E. coli* MoeB residue 155 is labeled with substitutions found in each structural class within each bar. PP = Polyproline motif of MoeBR and MoeZ proteins.

(BLAST E value < 0.01). Interestingly, *Ps-MoeZ* had higher similarity to seven sequences of mycobacterial, streptomycete, and cyanobacterial origin than to the most similar pseudomonad sequence (Table 2). NA BLAST searches showed that *Ps-moeZ* had higher homology, as measured by length of homologous overlap, to four of these same sequences than to pseudomonad sequences (Table 2, Fig-

ure 2a). We found that *Mlo-moeZdR*, *Mle-moeZ*, *Mt-moeZ*, *Pa-moeB*, *Pa-moeBR*, *Sc-moeBR*, and *Syc-moeZ* had significant homology (> 35 bp of > 75% identity) to *Ps-moeZ* (Table 2). To characterize the structure, function, and evolution of *Ps-MoeZ*, we chose to study these seven genes along with the *P. stutzeri* sequence. *Ec-moeB* was included in the study because its function has been extensively

Table 1: Sequences used in this study.

Organism	Strain	Designation	SSU rDNA	GenBank Accession Number		
				MoeB, BR, Z sequence		
				Protein	Nucleotide	Bases
<i>Escherichia coli</i>	K12	<i>Ec-moeB</i>		BAA35521	D90721	1398–2147 ³
<i>Mesorhizobium loti</i>	MAFF303099	<i>Mlo-moeZdR</i>	AP003001	BAB52000	AP003007	541–1293 ³
<i>Mycobacterium leprae</i>	TN	<i>Mle-moeZ</i>	AL583920	CAC30327	AL583919	277930–279117
<i>Mycobacterium tuberculosis</i> ¹	H37Rv	<i>Mt-moeBR</i>		CAB08373	Z95150	25473–26642
<i>Mycobacterium tuberculosis</i> ²	H37Rv	<i>Mt-moeZ</i>	Z83862	CAB08310	Z95120	2079–3257
<i>Nostoc</i> sp.	PCC 7120	<i>Nos-moeZ</i>	AP003587	NP_486946	AP003591	123713–124885 ³
<i>Pseudomonas aeruginosa</i>	PAOI	<i>Pa-moeB</i>		AAG08050	AE004880	4982–5740
<i>Pseudomonas aeruginosa</i>	PAOI	<i>Pa-moeBR</i>		AAG05491	AE004638	162–1340
<i>Pseudomonas stutzeri</i>	KC	<i>Ps-moeZ</i>	AF063219	AAF33130	AF196567	6475–7650
<i>Streptomyces coelicolor</i>	A3(2)	<i>Sc-moeBR</i>		CAC01308	AL390968	859–2037
<i>Synechococcus</i> sp.	PCC 7942	<i>Syc-moeZ</i>	AF132930	CAA76284	Y16560	37–1212
<i>Synechocystis</i> sp.	PCC 6803	<i>Syy-moeZ</i>	AL356612	BAA18438	D90914	48731–49909 ³

¹ The *moeBR* sequences of *M. tuberculosis* strains H37Rv, CDC1551, CSU#93, and 210 are identical. ² The *moeZ* sequences of *M. tuberculosis* strains H37Rv, CDC1551, CSU#93, and 210 are identical. ³ Coded by complement strand.

Table 2: Characteristics, similarities, and homologies of sequences used in this study.

Designation	Length		ThiF Domain BLAST ¹	RHOD Domain BLAST ¹	AA similarity to Ps-MoeZ			NA homology to Ps- <i>moeZ</i>	
	NA	AA			BLAST ²	Identity	AA Overlap	Identity ^{2,3}	Overlap (bp) ⁴
<i>Ec-moeB</i>	747	249	e-35	NA	e-42	39%	245	0	NA ⁵
<i>Mlo-moeZdR</i>	813	271	e-39	NA	e-66	52%	248	91%	35
<i>Mle-moeZ</i>	1185	395	e-40	e-10	e-121	57%	391	75%	474
<i>Mt-moeBR</i>	1167	389	e-43	e-13	e-103	50%	385	0	NA ⁵
<i>Mt-moeZ</i>	1176	392	e-39	e-8	e-121	57%	391	78%	486
<i>Nos-moeZ</i>	1170	390	e-42	e-9	e-95	49%	379	0	NA ⁵
<i>Pa-moeB</i>	762	254	e-30	NA ⁵	e-53	48%	245	80%	65
<i>Pa-moeBR</i>	1176	392	e-42	e-7	e-74	41%	389	79%	202
<i>Ps-moeZ</i>	1173	391	e-39	e-9	NA ⁵	NA ⁵	NA ⁵	NA ⁵	NA ⁵
<i>Sc-moeBR</i>	1176	392	e-34	e-10	e-114	54%	396	81%	325
<i>Syc-moeZ</i>	1173	391	e-42	e-9	e-101	53%	391	79%	286
<i>Syy-moeZ</i>	1176	392	e-31	e-8	e-93	47%	390	0	NA ⁵

¹ ThiF and RHOD domain E value exponential components reported as part of the CD results of standard protein BLAST (blastp) search. ² AA similarity and NA homology E value exponential components as reported by BLAST 2 sequences. ³ When genes had more than one segment of nucleic acid homology, the % identity values were determined by averaging the % identity of all segments. ⁴ When genes had more than one segment of nucleic acid homology, the lengths of these segments were added together. ⁵ NA = Not applicable.

characterized. *Mt-moeBR*, *Nos-moeZ*, and *Syy-moeZ* were included for reasons discussed later. BLAST searches of finished and unfinished microbial genomes [http://www.ncbi.nlm.nih.gov/Microb_blast/] yielded additional sequences in the genomes of *Corynebacterium diphtheriae*, *Mycobacterium avium*, *M. bovis*, and *M. smegmatis* with significant NA homology to *Ps-moeZ*, but these were not included in this study because of their potentially provisional nature, pending completion of the genome sequencing projects. Inclusion of these sequences in the analyses presented here did not change any of our conclusions.

Using each protein sequence listed in Table 1 as a query to search the Conserved Domain Databases, we found that each contained an N-terminal domain with high similarity (BLAST E value < 6⁻³⁰) to the ThiF family domain (Pfam00899) (Table 2, Figure 2c), suggesting that this portion of all the proteins in this study performed a similar function. The ThiF protein adenylates ThiS prior to sulfuration of that protein by a separate sulfurtransferase [23,24]. Pfam00899 encompasses the nucleotide binding site of ThiF. All 12 sequences in this region have high similarity to the derived consensus sequence GXXGXXG(7X)A(SX)GXXGXXG(4X)D(9X)R, which defines a nucleotide binding motif [16,25]. In addition, all sequences have five residues, or conservative substitutions in the case of Pa-MoeB, shown to participate in the adenylation reaction catalyzed by Ec-MoeB (Figure 3) [7]. The fact that such high similarities are retained in the ThiF coding region across three bacterial divisions suggests that this domain functions more or less autonomously. If the ThiF domain interacts only with dinucleotides and had evolved to a high degree prior to the speciation of the strains in our study, then there would be little selective pressure to fix additional modifications. The high degree of AA similarity that all 12 sequences have to the ThiF family domain probably indicates that these proteins, at least in part, perform a similar function, most likely activating a substrate by adenylation.

All of the predicted amino acid sequences except Ec-MoeB, Mlo-MoeZdR, and Pa-MoeB also have C-terminal similarity (BLAST E value < 1⁻¹²) to a rhodanese-like domain (Pfam00581) (Figure 2c, Table 2) [26]. Rhodanese, a thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1), is composed of two domains that are similar in three-dimensional structure but divergent in sequence [27]. All residues contributing to the active site of rhodanese are contained within its C-terminal domain. The cysteine that covalently bonds to the transferred S atom is conserved in all nine of the sequences that contain the rhodanese-like domain included in this study (data not shown). The similarity scores to the rhodanese-like domain, for those sequences that contain it, were lower than the ThiF

Ec-MoeB	-----	---MAELSD	QEMLRNROI	ILRGFDFDQ	EALKDSRVLII	VGLGLGCAA	46
Pa-MoeB	-----	-----MLSD	EELLRYSRQI	LLAQVDVGG	LRKRSRALI	VGLGLGSPV	44
Pa-MoeBR	-----	---MLATS	GGSGVEALSD	EEVQRVYRHI	ITLQVREGQ	QRLKQARVLI	55
Sc-MoeBR	-----	---MSLFP	LVEPAEALTV	DEVRYYSRHL	ILFDVGMGQ	KRLKNAKVLIA	55
Sc-MoeBR	-----	---MTEALI	PAKQISITRE	DEVRYYSRHI	ITLFDVGMQ	QRLKQARVLC	56
Mlo-MoeZdR	-----	---MTTLLTA	DEIERYARHI	ILPEVGGAG	QKQKQARVLI	IGAGGLGAPV	47
Syy-MoeZ	-----	---MLN	LDTDSIQLTQ	DDYARYSRHL	ILPEVGLGG	KRLKAAKVLIC	53
Nos-MoeZ	-----	---MLN	PNLEDIQLTK	DDYERYSRHL	ILPEVGGGQ	KRLKAAASVLC	53
Syy-MoeZ	-----	---MLN	PNLEAIQLTQ	DDYARYSRHI	ILPEVGLGG	KRLKAAASVLC	53
Ps-MoeZ	-----	---MPLSA	LVPAGELSR	AEINRYSRHL	ILFDVGMGQ	BRLKNAKVLIV	55
Mle-MoeZ	-----	---MSTSSLPP	LVEPAGLSR	DEVIRYSRHL	ILFDVGMGQ	MRLKARVLI	60
Mt-MoeZ	-----	---MSTSLPP	LVEPALSAR	EEVARYSRHL	ILFDVGMGQ	KRLKNAKVLIV	57
Ec-MoeB	SOYLASAGVG	NLLRLLDFDTV	SLSNLQROTLL	HSDATVGPQK	VEASARDLTR	INPHIATFPV	106
Pa-MoeB	ALYLAAGVG	ELHADFDTV	DLNLQROVVI	HDGASVGIK	VEASBRRLGA	LNQPIRLVSH	104
Pa-MoeBR	CLYLAAGVG	TIGLDFDVV	ESSNLQROVVI	HGYSITLMPK	VEASGRMMQD	INPLYEVTHV	115
Sc-MoeBR	LMYLAAGVG	TGLIIFDVEV	DESNLQROVI	HSQADIGRKP	AEASADTIQG	INPLVDVLIH	115
Mt-MoeBR	LLYLAAGVG	TIGIIGDGHV	DESNLQROVII	HGTSVGRPK	VEASAAEAV	INPHRVTVQ	116
Mlo-MoeZdR	LEVLAAGVG	TIGIVDDTV	SLSNLQROVI	HATDVGKIK	TDGARAATAH	INWVYVTH	107
Syy-MoeZ	LLYLAAGIG	RIGIVDFDVI	DHNSLQROVI	HGTSVGRPK	IEASAKRIHE	INPYCQVDLV	113
Nos-MoeZ	LLYLAAGIG	RIGIVDFDVV	DTSNLQROVI	HGTSVGRPK	IEASAKRIHE	INPYCQVDLV	113
Syy-MoeZ	LLYLAAGVG	RIGLVDFOIV	DTSNLQROVII	HGTSVGRPK	IEASAKSIME	INPCQVDLV	113
Syy-MoeZ	LLYLAAGVG	TIGIIGDFDV	DESNLQROVI	HGVDTVGLK	VDSAKKALAR	LNPFVQVETV	115
Mle-MoeZ	LLYLAAGVG	TIGIIGDFDV	DESNLQROVII	HGVADVGRSK	AQSARDSIVA	INPLVQRRLH	120
Mt-MoeZ	LLYLAAGVG	TIGIVDFDVV	DESNLQROVI	HGVADVGRSK	AQSARDSIVA	INPLVRLRHL	117
Ec-MoeB	NALLDDAELA	ALIAEHDVLV	DCTDNVAVRN	QINAGCFAK	VBLVSGAIR	MGGQITVFTY	166
Pa-MoeB	SNLDEEELD	ANAVARDVLV	DCTDNFATRE	AVNVACVAG	KFLVSGAIR	VEGASVDFP	164
Pa-MoeBR	RYAIDADNAQ	SLIQSYDLVL	DGTDNFATRY	LNVAVCAKLG	KPLVFGAIR	FEQOVSFNLV	175
Sc-MoeBR	QERLEADNVN	DIFSQYDLIV	DGTDNFATRY	LNVNACVLLN	KPYVWGSYR	FDQOASVFW	175
Mt-MoeBR	REMLTHDNAL	EIFGDHDLIV	DGTDNFATRY	LINDAVALAG	KPYVWGSYR	FNQTSVFWP	176
Mlo-MoeZdR	TFRLSADNAP	ALVTRFDIVV	DGSDNFATRY	VVADACASAR	KFLVRAAVGR	FDGQSVTVLK	167
Syy-MoeZ	ETRLSESNAL	SILRFPYDIV	DGTDNFATRY	LNVNACVLLG	KPNVYGSIFR	FEQOATVFNV	173
Nos-MoeZ	ETRLSESNAL	DIKFPYDIV	DGTDNFATRY	LNVNACVLLD	KPNVYGSIFR	FEQOATVFNV	173
Syy-MoeZ	ETRISANAL	DIAPYDIVI	DGTDNFATRY	LNVNACVLLN	KPNVYGSIFR	FEQOATVFNV	173
Ps-MoeZ	TDRLRERMAI	ELFSRYDILM	DGTDNFATRY	LNVNACVLLM	KPYVWGSIFR	FEQOASVFW	175
Mle-MoeZ	EFRLSESNVU	NLFQYDILV	DGTDNFATRY	LINDAVALAR	KPYVWGSYR	FEQOASVFW	190
Mt-MoeZ	ELRLASNAV	DLFQYDILIL	DGTDNFATRY	LNVNADVALG	KPYVWGSYR	FEQOASVFW	177
Ec-MoeB	ODGE-----	PCYRCLSRFL	G--ENALTCV	EAGVMAPLIG	VIGSLQAMEA	IHMLAGYKPK	218
Pa-MoeB	RRDES-----	PCYCHLYGHG	SE--EALITCS	EAGVIGPLVG	LVGSLQALEA	LKLLAGFGEF	217
Pa-MoeBR	EGG-----	PCYCLEFNNI	PFPEMAPSCN	AGGVIGPLFG	VIGLILQATEA	VKALVGLGRS	228
Sc-MoeBR	EHG-----	PCYRCLPEP	PFPEMAPSCA	EGGVGLGCA	SIGSIQTNEA	IKLLGGIPEP	228
Mt-MoeBR	GRG-----	PCYRCLHPAP	PFPEGLVPSCA	EGGVGLGCA	TIASIQVTEV	LKLLGGVTEP	229
Mlo-MoeZdR	FEYKDGKPN	PGVRLDFPEA	PFPEGLVPSCA	EAGVIGALGT	VVGTIQAMEA	IKLITGTGEP	227
Syy-MoeZ	EDG-----	PNRDLYPEP	PFPEMAPSCA	EGGVGLGPG	IIGVIGQATE	VKTLIQSQT	226
Nos-MoeZ	EGG-----	PNRDLYPEP	PFPEGLVPSCA	EGGVGLGPG	IIGVIGQATE	VKTLIQSQT	226
Syy-MoeZ	QGG-----	PNRDLYPEP	PFPEMAPSCS	EGGVGLGPG	VVGTIQATEA	LKILIGAPNT	226
Ps-MoeZ	NAPNDLG---	LNVRDLYPEP	PFPEMAPSCS	EGGVGLGCA	SIASIMATEA	VKILITGTGEP	232
Mle-MoeZ	DAPDGLG---	LNVRDLYPEP	PFPEMAPSCA	EGGVGLVYCA	SIASVMGTEA	IKLITGTGAP	237
Mt-MoeZ	DAPDGLG---	LNVRDLYPEP	PFPEMAPSCA	EGGVGLGCA	SVASVMGTEA	IKLITGTGEP	234
Ec-MoeB	ASGKIVMYDA	MTCQFREMKL	MRNPCEVCG	Q			249
Pa-MoeB	LVGRLLLVDA	LGTFRFELRV	RRDPSACVCG	QRHG			252
Pa-MoeBR	LSGRLLRFDA	LMHFNFVKF	NRDDCPACG	KTAANFRHW	REGLLCOAS-	-AAPSRLDD	286
Sc-MoeBR	LVGRLLMYEA	LEMVYKQVI	RQDPCVAVCG	ENPFTLELD	VEAFQCV---	VEEAGAAA	285
Mt-MoeBR	LVGRLLMYEA	LDATYHQIRI	AKNDFCAICG	DAPITTELD	DSVSC-----	--ASTQSDP	282
Mlo-MoeZdR	LIGRLLLVDA	LAARFETRY	SRR				250
Syy-MoeZ	LSGRLLLVNA	LDMPFRELKI	RFNP-----	ERPVIDRLID	YQEFQGIQPA	QAEEAATDS	280
Nos-MoeZ	LSGRLLLVNA	LDMPFRELKI	RFNP-----	TRFVIEKILID	YEQFCGIQPA	KAEAEQWDE	280
Syy-MoeZ	LSGRLLLVNA	WDMFRELKI	RFNP-----	VSRVIEKILD	YQFCGIQPA	KAEAEQASA	280
Ps-MoeZ	LLGRLLVVDA	LDMRVRELFP	RRLNP-----	RQPITDLAED	YQVFCGL---	GLPKGDADA	284
Mle-MoeZ	LLGRLLMYNA	LEMSYRRIRI	HKDP-----	SRKPIETELD	YQFCGCV---	VSDDAQVAT	288
Mt-MoeZ	LLGRLLVVDA	LEMSYRTITI	RKDP-----	STFKITTELD	YEQFCGCV---	VADDAQAQAK	285
Ec-MoeB							
Pa-MoeB							
Pa-MoeBR	DRYI 290						346
Sc-MoeBR	DSTI 289						344
Mt-MoeBR	ELVI 286						341
Mlo-MoeZdR							
Syy-MoeZ	IPEI 284						340
Nos-MoeZ	IQEM 284						340
Syy-MoeZ	LSDM 284						340
Ps-MoeZ	VQGI 288						343
Mle-MoeZ	GSTV 292						347
Mt-MoeZ	GSTI 289						344

Figure 3 Alignment of sequences included in this study. The entire lengths of the MoeB and MoeZdR proteins and all except the rhodanese domains of MoeBR and MoeZ proteins are included. The existence rhodanese-like domains (RHOD) are indicated by lines for the sequences that include them. Positions of CXXC motifs are marked with vertical arrows. Positions of Ec-MoeB active sites residues that participate in adenylation reactions are marked with filled circles. Position 155 of Ec-MoeB is highlighted with a star. The polyprotein motif found in MoeBRs and MoeZs is boxed.

similarity scores. However, since the rhodanese domain is characterized by conservation in terms of three dimensional structure rather than primary sequence, these lower similarity scores are not surprising.

Examination of the Clustal X-alignment of all the protein sequences revealed significant structural differences (Figure 3). In addition to the ThiF family domain, all sequences except Mlo-MoeZdR also possessed a region similar to the C-terminal half of ThiF. Ec-MoeB, Mt-MoeBR, Pa-

MoeB, Pa-MoeBR, and Sc-MoeBR all contain two pairs of cysteine residues, each arranged in a CXXC motif and spaced 68–70 residues apart, like those in ThiF proteins (arrows in Figures 2c and 3). But the Ps-MoeZ sequence, along with Mlo-MoeZdR, Mle-MoeZ, Mt-MoeZ, Nos-MoeZ, Syc-MoeZ, and Syy-MoeZ, lacks these four cysteine residues. In all these sequences except Mlo-MoeZdR, the first CXXC motif is replaced with NYRD. In Mlo-MoeZdR the first CXXC is substituted with GYRD. All these sequences except Mlo-MoeZdR also have a gap in place of the second CXXC. Mlo-MoeZdR ends prior to the position where the second CXXC would occur. This dual CXXC motif, found in MoeB, ThiF, and HesA proteins, forms a metal center with a zinc atom [7,9]. The presence of all four of these cysteines has been shown to be required for activity in MoeB). [5]. Therefore, the proteins lacking them are unlikely to function as MSAEs. We designated the region containing these two cysteine pairs as 2X CXXC for the purposes of our discussion (Figure 2C).

MoeBR, MoeZdR, and MoeZ proteins can be further differentiated from MoeBs by the inclusion of a polyproline motif immediately following the first CXXC location (boxed area in Figure 3). This polyproline tract is immediately adjacent to a disordered surface loop found in MoeB proteins thought to be involved in protein-protein interactions [7]. The consensus for this motif (RXLYPEPPPP) closely corresponds to a sequence (RSLYPPPPPP) found in rat and human metabotropic 1 glutamate receptors [28,29]. This motif is reminiscent of protein-protein interaction domains found in some proteins [30]; however, interactions of this type have not been shown to be facilitated by the polyproline motif in rat and human metabotropic 1 glutamate receptors.

An AA substitution found in MoeBRs and MoeZs may alter the substrate specificity of these enzymes. In Ec-MoeB, β sheet 5 (residues 149–155 of Ec-MoeB, Figure 3) forms part of the pocket which accepts the C-terminal portion of MoaD, of which the terminal glycine is subsequently adenylated [7]. Ec-MoeB I155, located at the C-terminal end of this beta loop, is substituted in MoeBR proteins by Y and in MoeZ proteins by F or Y. These bulky substitutions may restrict access of the C-terminus of MoaD-like proteins and may indicate that a different type of substrate is adenylated by MoeBR and MoeZ.

The presence or absence of two features, the two pairs of cysteines in the 2X CXXC domain and the rhodanese-like domain, form the primary basis for differentiation between the four different protein structures among the sequences found in Table 1 (Figure 2c). MoeB, as represented by the functionally characterized Ec-MoeB, consists of an N-terminal ThiF domain followed by a 2X CXXC domain with both CXXC pairs intact and no rhod-

anese-like domain. MoeBs also lack the polyproline motif and have I at position 155. MoeBR has the ThiF and 2X CXXC domains configured like MoeB but includes an N-terminal rhodanese-like domain, the polyproline motif, and has a Y substitution at position 155 (Ec-MoeB numbering). MoeZ has all the components of MoeBR, but all the cysteines in the CXXC motifs are absent and position 155 contains either F or Y. Finally, MoeZdR (MoeZ, deleted Rhodanese) contains the ThiF domain followed by an N-terminal portion of the 2X CXXC domain that has GYRD substituted for the first CXXC and terminates prior to the second CXXC pair. (MoeZdR probably does not function as a MoeB, BR, or Z protein because of its substantial truncation. It is included in this study only because of its homology to Ps-MoeZ.) Once these structural definitions were defined, we decided to add Nos-MoeZ and Syy-MoeZ to our data set since they were MoeZs that we had not included in our dataset by reason of homology. We also included Mt-MoeBR because we were interested in the relationships between MoeBR and MoeZ sequences that were coresident in the same genome.

The Conserved Domain Database was screened for additional sequences related to the Ps-MoeZ sequence. This search yielded 19 proteins structurally similar to MoeBR and MoeZ proteins. Five of these were eukaryotic sequences of unknown function; five others were eukaryotic MSAE proteins; and one was a ThiF ORF within an operon containing two other thiamin biosynthesis genes in *Erwinia amylovora* [31]. The remaining eight sequences were of bacterial origin and were already included in our dataset. A similar search using Pa-MoeB as the query yielded 146 sequences with a domain structure similar to MoeB, most of which were entered into the database as MoeB, ThiF, HesA, or ubiquitin-activating enzymes from archaea, bacteria, and eukaryota. In terms of distribution, the MoeB structure (146 sequences) was much more common than the MoeBR structure (12 sequences), while MoeZ was represented by only six sequences in the GenBank non-redundant (nr) database.

Based on our sequence analysis, some *moeZ* sequences have been misidentified. Syc-MoeZ, Syy-MoeZ, and Nos-MoeZ are identified as a MoeBs in GenBank accession numbers CAA76284, BAA18438, and NP_486946, respectively. The lack of all essential cysteines in both copies of the CXXC motifs suggest that these MoeZs probably do not function like MSAE proteins.

Conserved NA sequences

When the regions of greater than 75% NA homology between each individual NA sequence and *Ps-moeZ* were plotted, it became apparent that these homologies clustered in defined regions (Figure 2a). Five sequences had a homologous region within the N-terminal half of the ThiF

domain; six sequences had a homologous area in the C-terminal half of the same domain; and four sequences showed homology to areas of the 2X CXXC domain. Five sequences with greater than 200 bp of more than 75% NA acid identity to *Ps-moeZ* (*Mle-moeZ*, *Mt-moeZ*, *Pa-moeBR*, *Sc-moeBR*, and *Syc-moeZ*) along with *Ps-moeZ* were designated the "high homology group" and used in subsequent analyses of the homologous regions.

Using a codon-based alignment of the NA sequences in the high homology group, we analyzed the occurrence of synonymous and nonsynonymous substitutions over the entire coding region. The plot of the ratio of synonymous/nonsynonymous substitutions (dS/dN) identified five regions where the synonymous substitution rate exceeds the nonsynonymous substitution rate ($dS/dN > 1$), suggesting that homology in these areas was driven by the need for AA conservation (Figure 2b). Analysis of a plot of the degree of identical amino acid residues among the high homology group showed that a high degree of AA similarity existed in these same regions (data not shown). Visual inspection of the NA alignment of the high homology group revealed that substitutions in the areas where dS exceeded dN tended to involve the second and third bases of the codons, confirming the previous suggestion (data not shown). There was also a correlation between the regions of homology to *Ps-moeZ* and the areas where synonymous substitutions exceeded nonsynonymous substitutions (Figure 2a and 2b). These correlations support the supposition that the NA homologies among the sequences are driven by functional constraints of the proteins and not by the need to maintain secondary nucleic acid structure. Analysis of predicted secondary mRNA structure did not reveal any conserved stems or loops in the regions of NA conservation among the high homology group (data not shown).

Function

The functionality of MoeB, MoeBR, and MoeZ are known. MoeB, exemplified by Ec-MoeB, and MoeBR, exemplified by cnxF of *Aspergillus nidulans* [16], participate in the synthesis of MPT-Mo as MSAEs. This is a housekeeping function for most organisms. MoeZ, on the other hand, has been shown to be necessary for the synthesis of pdtc, an unusual metabolite. However, since CTN1, a mutant of *P. stutzeri*, missing a 171 kb genomic fragment that includes *moeZ* [17], reduces nitrate as does the wild-type (data not shown), it is evident that *Ps-moeZ* is not necessary for MPT-Mo synthesis.

Both *moeBR* and *moeZ* coexist in the *M. tuberculosis* genome, which exhibits nitrate reductase activity, but *moeBR* is missing from *M. leprae*, which has no nitrate reductase activity. The fact that these two separate genes are maintained in *M. tuberculosis* supports the supposition that

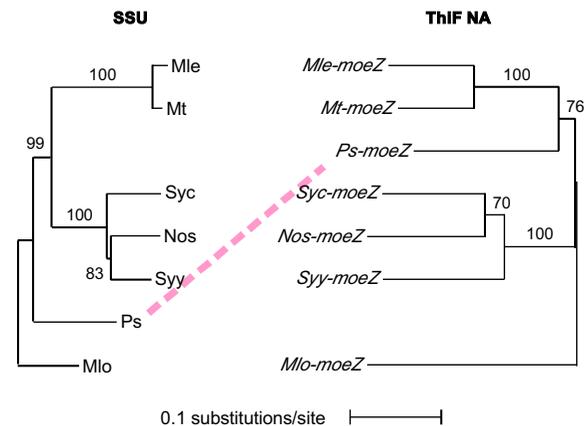


Figure 4

Maximum likelihood, neighbor joining trees of *moeZ* Thif domain and associated SSU sequences used in this study. The incongruent placement of *P. stutzeri* between the trees is highlighted with a dotted line. Confidence estimates are placed near the branches that they apply to.

they perform different functions. On the other hand, the sequencing of the nitrate-reducing cyanobacteria *Synechocystis* sp. PCC6803 and *Nostoc* sp. PCC7120 is complete, but no MSAE sequences have been found. Perhaps MoeZ fulfills MSAE function as part of a modified MPT-Mo pathway in these species.

MoeBR incorporates a rhodanese-like domain, but MoeB does not. Examples of both MoeB (*E. coli*) and MoeBR (CNXF of *A. nidulans*) have been characterized as MSAEs, indicating that the C-terminal rhodanese-like domain addition does not inhibit MSAE activity. The presence of a rhodanese domain in MoeBR and MoeZ suggests that these members of the family may perform an additional function compared to MoeB. *P. aeruginosa* has both a *moeB* and a *moeBR*, also suggesting that the products of these two genes have different functions or perform the same function in a slightly different manner. However, there is no physiological evidence for the functional significance of the rhodanese domain in MoeBR. Rhodanese extracts a sulfur from sulphane sulphur containing anions (e.g., thiosulfate), forms a temporary covalent linkage between the S atom and a conserved cysteine residue, then transfers the sulfur to a thiophilic acceptor (e.g., cyanide). Whether sulfur transferase functionality is retained in the rhodanese-like domains of MoeBR and MoeZ and whether these proteins can function to activate a substrate as well as transfer a sulfur-containing moiety is unknown.

Evolution

Because of the definitive structural features shared among MoeZs and the homologies among their coding regions,

we decided to investigate the phylogenetic relationships of *moeZ* sequences. To infer baseline interspecies evolutionary relationships we performed a phylogenetic analysis of the 16S rDNA (SSU) sequences from the seven *MoeZ*-containing strains included in our study. We obtained sequences for all SSU alleles for each strain from GenBank. Analysis of all the SSU alleles for strains with multiple copies revealed that there was no substantial variation among them. We chose one representative full-length SSU sequence for each strain for our analyses (Table 1). The tree of SSU sequences shows the two proteobacteria (*P. stutzeri* and *M. loti*) at one end of the tree with cyanobacteria (*Synechococcus* sp. and *Synechocystis* sp.) and mycobacteria (*M. leprae* and *M. tuberculosis*) clades branching off from a common node (Figure 4). This tree topology agrees with those that have been previously published [32].

The *moeB/BR/Z* sequences under study are a mixture of orthologs and paralogs, and a combined study of their phylogeny is not possible, so we limited our study to *moeZ* sequences. We constructed the tree from a gapless region of the NA alignment spanning the entire ThiF domain except for the last two bases. The topology of the ThiF domain tree matches the SSU tree except that *Ps-moeZ*, which was adjacent to *M. loti* in the SSU tree, appeared between the cyanobacteria and mycobacteria clades (Figure 4). Parsimony, likelihood, and distance methods all resulted in trees with the same topology. Moreover, the placement of *Ps-moeZ* in the ThiF tree was not changed when full-length *MoeZ* AA sequences were analyzed using the same methods.

Additional support for the topology of the ThiF domain tree was sought by conducting a manual analysis of the insertions and deletions (indels) in the alignment of full-length *moeZ* sequences. *Ps-moeZ* contains five indels in the codon-based NA alignment (Table 3). Of these, one is unique to *Ps-moeZ*; four are shared with members of the mycobacteria clade; and none are shared with members of the cyanobacteria clade or *Mlo-moeZdR*. Thus the results of the indel analysis supported the position of *Ps-moeZ* in the ThiF domain tree.

The incongruence between the SSU and the ThiF domain trees suggests that *Ps-moeZ* may have been acquired by *P. stutzeri* strain KC by lateral gene transfer (LGT) from an ancestor more closely related to mycobacteria and cyanobacteria than to proteobacteria. The results of the indel analysis also suggest that *Ps-moeZ* is more closely related to the mycobacteria *moeZ* sequences than to the cyanobacteria and proteobacteria *moeZ* sequences. BLAST homology results also support this close relationship to the mycobacteria.

Although they were not included in the analysis presented here, the homologies of *Ps-moeZ* to sequences in the unfinished microbial genomes of *M. avium*, *M. bovis*, and *M. smegmatis* were also very high and thus provide additional support for the placement of *Ps-moeZ* in the ThiF tree and suggest that the direction of LGT was from a mycobacterial species to *P. stutzeri*.

Comparison of the GC/AT percentages and codon bias profiles of *Ps-moeZ* to data from other known *P. stutzeri* strain KC coding regions indicates that acquisition of *Ps-moeZ* was ancient. The GC/AT percentages for the 1st, 2nd, 3rd, and all three codon bases combined compiled from the seven published coding regions of *P. stutzeri* strain KC were 66.0%, 46.8%, 72.2%, and 61.7%, respectively. For *Ps-moeZ*, these values were similar (64.8%, 42.6%, 75.3%, and 60.9%, respectively). A comparison of the *Ps-moeZ* codon bias table with the one compiled from the seven published strain-KC coding regions did not reveal any divergent trends. Many of these substitutions could be due to GC/AT percentage adjustment to the *P. stutzeri* genome, but since the donor is unknown and only seven ORFs have been sequenced from strain KC, a definitive analysis is not possible.

It is not unreasonable that a pseudomonad coexisting in the soil with cyanobacteria, mesorhizobium, streptomycete, and free-living mycobacteria could acquire *moeZ* from one of these species. *P. stutzeri* has been characterized as having an exceptionally plastic genome with high genotypic diversity within the species [33]. LGT events are thought to contribute to this plasticity and, consistent with this, many *P. stutzeri* strains also exhibit a natural transformation ability [34]. The fact that incorporation of DNA into the genome through homologous recombination requires a region of homology usually limits LGT mediated by natural transformation to closely related species [34,35]. The ThiF domains of the high homology group do provide a region of homology suitable for homologous recombination. This homology is shared across a wide range of species and thus could have contributed to a natural transformation-mediated LGT event in *P. stutzeri*. This mechanism of acquisition is supported by the fact that no phage genes, transposase coding sequences, IS elements, or repeat sequences were found in the 25.7 kb cosmid clone that contains *Ps-moeZ* [17].

Three other *P. stutzeri* isolates, obtained from the American Type Culture Collection, have been shown by Southern analysis to lack *pdtc* synthesis genes. These same strains plus *P. aeruginosa* PAO1, *P. putida* mt2, and *P. fluorescens* F113 were also shown not to produce *pdtc* [17]. *Pdpc* production in bacteria other than *P. stutzeri* strain KC is limited to *P. putida* strain To8 and one other unclassified pseudomonad strain [36]. *P. stutzeri* and *P. putida* are

Table 3: Indel analysis of MoeZ NA sequences. (X indicates that indel is present.) Numbering begins with the first base of Mle-moeZ.

Indel Number	1	2	3	4	5	6	7	8	9	10
Begin (bp)	555	564	795	798	864	948	1086	1065	1098	1182
Length (bp)	21	9	18	15	9	3	15	6	3	3
<i>Mlo-moeZdR</i>										
<i>Mle-moeZ</i>		X	X		X	X	X			
<i>Mt-moeZ</i>		X	X		X	X	X			
<i>Ps-moeZ</i>		X		X	X	X	X			
<i>Syc-moeZ</i>	X		X						X	X
<i>Nos-moeZ</i>	X		X					X		X
<i>Syy-moeZ</i>	X		X							

closely related, both belonging to rRNA homology group I [37], so they may be descendants of the original pseudomonad that acquired *moeZ* by LGT. The relative rarity of pdtc production among pseudomonads, especially the *P. stutzeri* strains, is consistent with the hypothesis that *Ps-moeZ* was acquired through LGT by strain KC. Alternatively, a pseudomonad ancestor could have contained the genes for and produced pdtc, but this function could have been lost from most strains. Loss of this function is plausible if pdtc production is a highly specialized function or is only required for survival in extreme or competitive environments.

Conclusions

It is not known what function MoeZ performs in the mycobacteria and cyanobacteria species that contain it. Indications are that in mycobacteria, MSAE function is performed by MoeBR. But in the fully-sequenced cyanobacteria there are only MoeZs and no MSAE, so in these organisms MSAE function may be fulfilled by MoeZ. In either case, MoeZ is essential to pdtc synthesis in *P. stutzeri* strain KC, and pdtc is a potent and versatile molecule. Its capabilities of enhancing iron uptake and iron reduction make it a good candidate for a pathogenicity factor. The role of MoeZ in the pathogenic mycobacteria and its impact on virulence of these strains should be investigated.

Materials and Methods

Sequences were obtained from the GenBank non-redundant (nr) sequence database at the National Center for Biotechnology Information. BLAST searches were used to obtain the initial set of sequences similar to *P. stutzeri* strain KC ORF-F [38]. The program, BLAST 2 Sequences, was used to obtain the degrees of similarity and homology among amino acid (AA) and nucleic acid (NA) sequences [39]. Protein domains, as codified by Pfam [26], were

identified by searching the Conserved Domain Database using the Domain Architecture Retrieval Tool provided by GenBank.

Protein alignments were generated using the Clustal X program and checked manually [40]. SSU NA sequences were aligned using Clustal W as implemented by the OMI-GA package and checked manually [41,42]. NA alignments of full-length *moeZ* sequences for indel analysis were aligned manually based on the Clustal X protein sequence alignment.

Codon bias tables and GC/AT percentages were calculated using the Countcodon program available from the Codon Usage Database [43].

Synonymous and non-synonymous substitution rates were calculated by the Synonymous/non-synonymous Analysis Program (SNAP) provided by Los Alamos National Laboratory [http://hiv-web.lanl.gov/] using the codon-based NA alignment of the high homology group. Substitution rates were averaged over an 18 codon window to smooth the data for graphing.

Phylogenetic trees were inferred using PAUP 4.0 [44]. Three data sets were analyzed: those portions of the NA alignment coding for the ThiF domains of the MoeZ conceptual proteins, the alignment of the complete MoeZ sequences, and the alignment of the full length 16S rDNA (SSU) sequences. Maximum likelihood, parsimony, and distance methods were used for analysis. Confidence estimates were determined by bootstrap analysis based on 1000 replicates.

RNA secondary structure of the ThiF domain coding regions of the sequences in the high homology group was

predicted using the GeneBee web server [http://www.genebee.msu.su/] using both individual sequences as well as alignments [45].

Authors' Contributions

MSC conceived and designed the study, performed the molecular genetic analyses, produced the sequence alignments, and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank Sarah Koerber for her editorial assistance. This material is based on work supported by, or in part by, the U.S. Army Research Laboratory and the U.S. Army Research Office under the contract/grant number DAAD19-00-1-0078.

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