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The MarR Family of Transcriptional Regulators – A Structural Perspective

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1. Introduction

All living organisms have molecular systems that enable them to resist a variety of toxic substances and environmental stresses. Proteins belonging to the **M**ultiple **a**ntibiotic **r**esistance **R**egulators (MarR) family reportedly regulate the expression of proteins conferring resistance to multiple antibiotics, organic solvents, household disinfectants, oxidative stress agents and pathogenic factors (Alekshun & Levy, 1999a; Miller & Sulavik, 1996; Aravind et al., 2005). The *marR* gene was initially identified as a component of the negative regulator encoded by the *marRAB* locus in *Escherichia coli* (George & Levy, 1983a, b). Currently, a large number of MarR-like proteins (~12,000) can be found in bacterial and archaeal domains, and the physiological role of around 100 of them have been characterized. Members of the MarR family of transcriptional regulatory proteins form a homodimer to bind to their cognate double-stranded DNA (dsDNA). The protein-DNA interactions is regulated by specific phenolic (lipophilic) compounds, such as salicylate, ethidium, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and benzoate. The MarR homologues contain a winged helix-turn-helix (wHtH) motif at the DNA binding site, and this motif is well known for DNA binding in eukaryotes, prokaryotes, archaea and viruses. In this chapter, we will discuss the identification, three-dimensional structure and interactions with ligand (drug)/DNA of MarR family proteins.

2. Identification and characterization of MarR family proteins

The MarR family of transcriptional regulators was first identified in multidrug resistant strains of *E. coli* K-12 (George & Levy, 1983a, b). This MarR protein plays a key role in regulating the multiple antibiotic resistance (*marRAB*) regulon, which is responsible for the mar phenotype manifesting as resistance to a variety of structurally and medicinally important antibiotics, including sodium salicylate, tetracycline, chloramphenicol, penicillins, β-lactams, puromycin, fluoroquinolones and organic solvents (Cohen et al., 1993a). The *marA* gene encodes a transcriptional regulatory protein MarA, which is a member of the AraC protein family. As an activator of the *marRAB* operon, MarA induces the expression of over 60 genes responsible for the mar phenotype, including the AcrAB-TolC multidrug efflux system (Alekshun & Levy, 1997; Okusu et al, 1996). The *in vivo* upregulation of *marRAB* expression and the mar phenotype have been experimentally shown to be activated

by a wide range of antibiotics and phenolic compounds, such as 2,4-dinitrophenol, menadione, plumbagin and salicylate (Cohen etal., 1993b; Seoane & Levy, 1995).

Similar to MarR, MexR negatively regulates an operon in *Pseudomonas aeruginosa* that, when expressed, encodes a tri-partite multidrug efflux system that results in increased resistance to multiple antibiotics, including tetracycline, β-lactams, chloramphenicol, novobiocin, sulfonamides and fluoroquinolones (Li & Poole, 1999; Srikumar et al., 2000). Analysis of the open reading frame of *mepA* reveals that the gene is part of the *mepRAB* three gene cluster, which encodes MepR, a MarR family member. MepR binds to compounds like ethidium, DAPI and rhodamine 6G. Some members of the MarR family of DNA-binding proteins, such as hypothetical uricase regulator (HucR) and organic hydroperoxide resistance regulator (OhrR), mediate a cellular response to reactive oxidative stress (ROS) (Wilkinson & Grove, 2004; 2005). The *Deinococcus radiodurans* HucR was shown to repress its own expression as well as that of a uricase. This repression is alleviated both *in vivo* and *in vitro* upon binding uric acid, the substrate for uricase. As uric acid is a potent scavenger of reactive oxygen species, and *D. radiodurans* is known for its remarkable resistance to DNA-damaging agents, these observations indicate a novel oxidative stress response mechanism (Hooper et al., 1998; Kean et al., 2000; Ames et al., 1981). Similar to HucR, the OhrR protein of *Bacillus subtilis* also mediates a response to oxidative stress; however, for OhrR, it is oxidation of a lone cysteine residue by organic hydroperoxides that abrogates DNA binding (Fuangthong et al., 2001; Fuangthong & Helmann, 2002).

2.1 Crystal structure of MarR homologues

Recently, much structural information have become available for MarR homologues. The MarR proteins exist as homodimers in solution, and as mentioned above each monomer consists of a wHtH DNA binding motif. We have recently solved one of the MarR regulators, ST1710 in the absence (apo)/presence (complex) of salicylate and in the presence of the putative DNA promoter. The overall structure of ST1710 indicates that it belongs to the α/β family of proteins and resembles those of the MarR family of proteins. It consists of six α-helices and two β-strands, arranged in the order of α1-α2-α3-α4-β1-β2-α5-α6 in the primary structure. The asymmetric subunit contains one molecule of ST1710. Two monomers of ST1710 are related by a crystallographic 2-fold symmetry to form the dimer, and this is consistent with our gel-filtration analysis (Kumarevel et al., 2008) as well as with other MarR family proteins (Alekshun et al., 2001; Lim et al., 2002; Liu et al., 2001; Wu et al., 2003; Hong et al., 2005) (Fig. 1). The N- and C-terminal residues located at the helices of each monomer are closely intertwined and form a dimerization domain, which is stabilized by hydrophobic and hydrogen bonding interactions between the residues located within these regions. Apart from the dimerization domain, as observed in many DNA binding transcriptional regulators, the residues located within the α2-α3-α4-β1-β2 structure form the wHtH DNA binding motif (Alekshun et al., 2001; Hong et al., 2005; Bordelon et al., 2006; Newberry et al., 2007; Saridakis, et al., 2008). The residues involved in dimerization play a key role in maintaining the distance between the DNA recognition helices in the wHtH loops, which can ultimately affect the fidelity and strength of the protein-DNA interactions. Mutagenesis of the residues involved in the dimeric interface has been shown to cause low DNA binding affinity (Andresen et al., 2010). Furthermore, C-terminal deletion in MarR homologs decreases the ability to form dimers, which correlates with the attenuated DNA binding affinity and increased phenotypic resistance in *E. coli* (Linde et al., 2000).

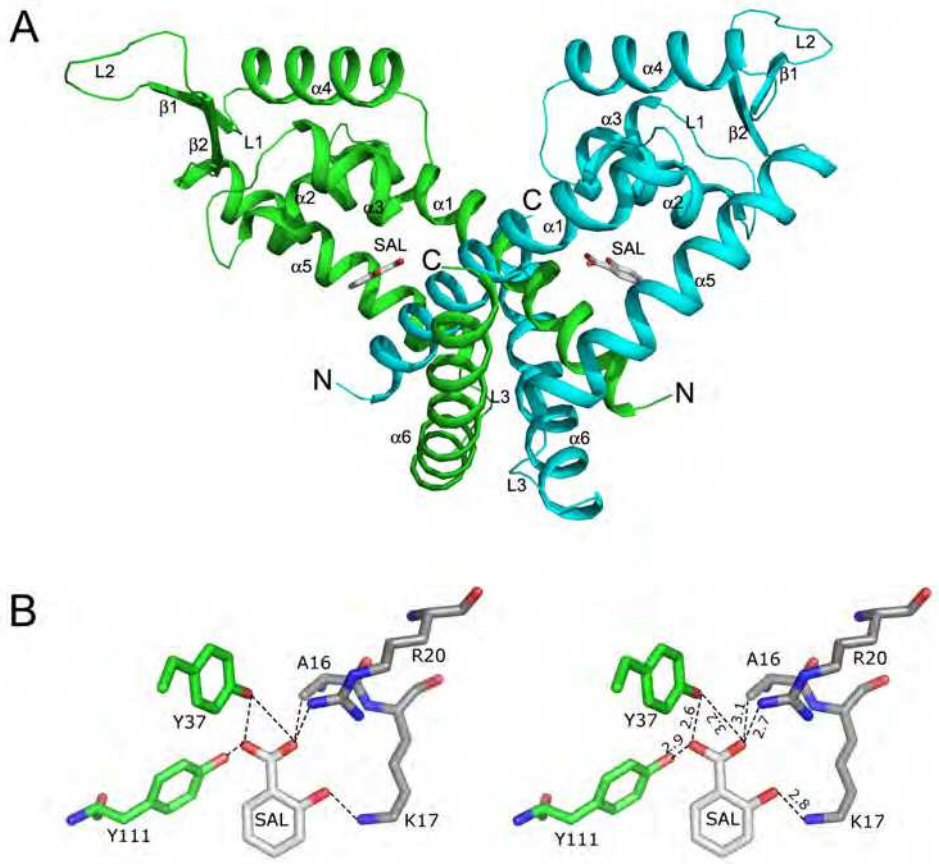
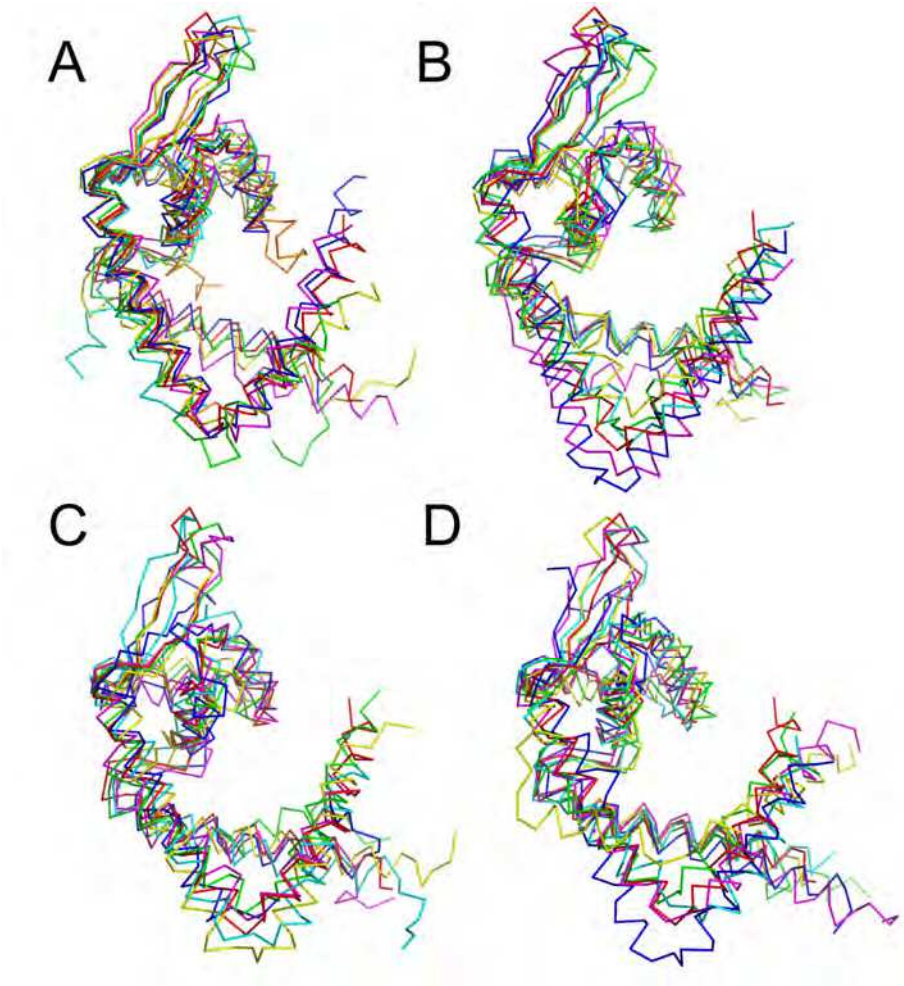


Fig. 1. Crystal structure of ST1710, a member of MarR family proteins. (A) A ribbon diagram of ST1710-salicylate complex dimer is shown. The secondary structure assignments and the N- /C-termini are labeled on the structure. (B) Close-up stereo view of salicylate binding site interactions with protein residues is shown. The hydrogen bonds are indicated by broken lines.

2.2 Structural comparison of MarR homologues

In a search for proteins with structural similarity to ST1710 protein within the known structures available in the Protein Data Bank (www.pdb.org) using the Dali program (Holm and Sander, 1996), we have identified many other protein structures within the MarR superfamily with good Z-scores. The highest ranked among those proteins is a Syla-like protein from *Enterococcus faecalis* (pdb id, 1lj9, Z-score=17.7, sequence identity=22%), which has been shown to up-regulate the expression of molecular chaperones, acid-resistance proteins and cytolysin, as well as to down-regulate several biosynthetic enzymes (Wu et al., 2003). The second highest ranked protein is a hypothetical regulator from *P. aeruginosa* (pdb ids, 2fbh, 2nnn, 2fbi), and the third one is OhrR from *B. subtilis*, an organic hydroperoxide-

resistance regulator that controls the expression of the organic hydroperoxide resistance (*ohr*) gene by binding to *ohrA* promoter elements (Hong et al., 2005). Many proteins (1jgs, 1s3j, 2a61, 2nyx, 2hr3, 1xma, 3f3x, 2eth, 3nqd, 3nrv, 3bpv, 3bpx, 3s2w, 3deu, 3q5f, 3fm5, 3oop, 3cdh, 3cjn, 3e6m, 3k0l, 3bro, 3eco, 3jw4, 3bj6, 3g3z, 1lnw, 3bja, 3qww, 3kp6, 3bdd, 1z91, 2pex, 2bv6, 3hrm, 1ub9) were identified with Z-scores between 10-16. All of these proteins adopt a similar topology (rmsd between 1 to 4 Å), despite the low (~15-25%) sequence identities between them, and these sequence dissimilarities are reflected throughout the secondary structural elements (Figs. 2, 3). In addition, the high flexibility of the DNA binding domains displayed in the different crystals provides indirect evidence of the ability of this wHtH motif to adapt in order to recognize various DNA targets. In addition, a sequence homology search against ST1710 (Q96ZY1 from *Sulfolobus tokodaii*) in the non-redundant protein database using fasta revealed that many archaeal species have conserved motifs resembling MarR family regulatory sequences, including *Sulfolobus acidocaldarius*, *Sulfolobus solfataricus*,



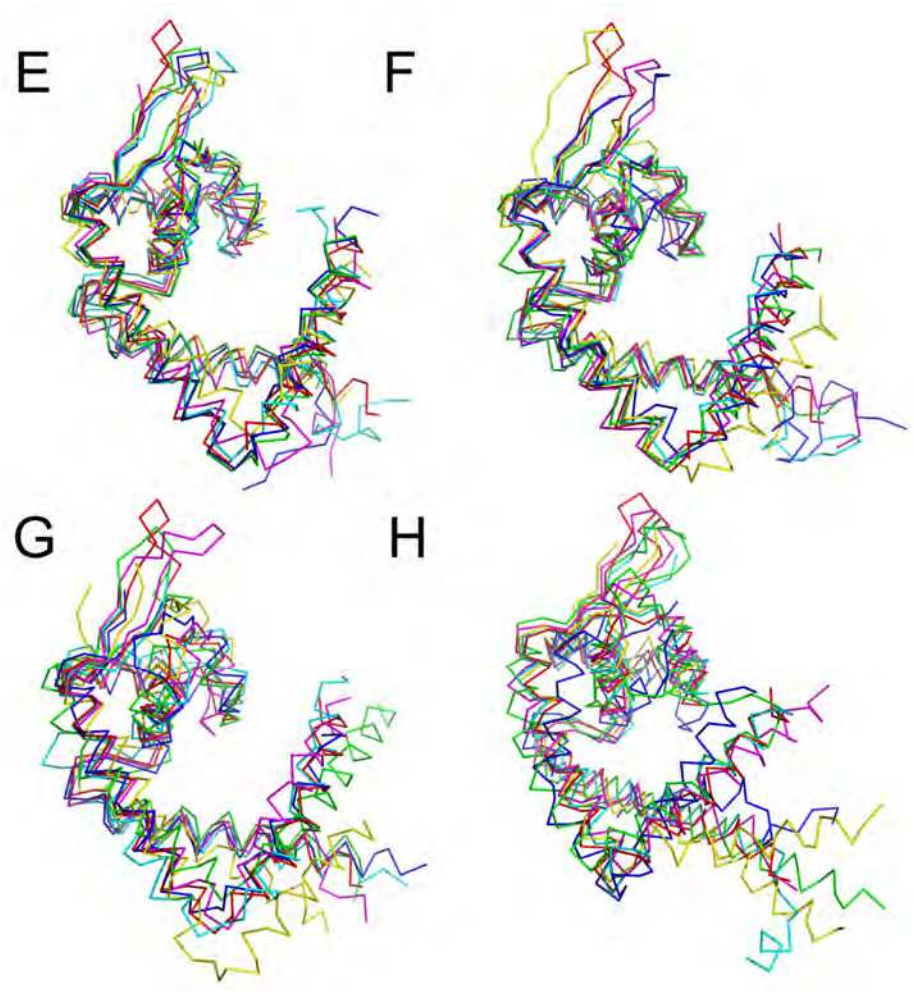
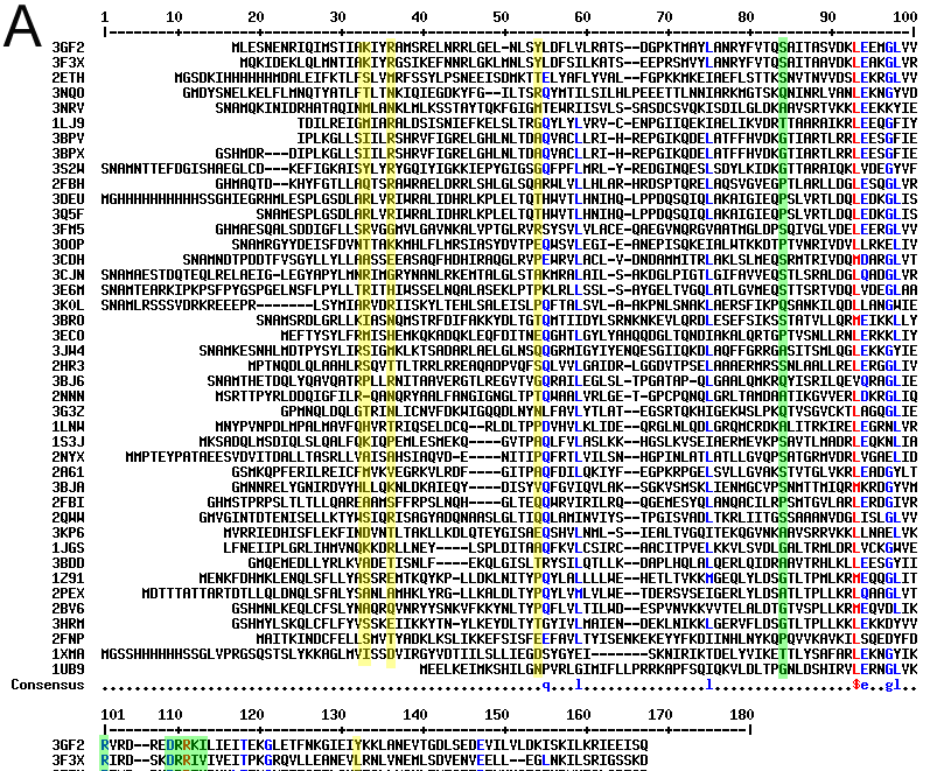


Fig. 2. Three-dimensional structural comparison of ST1710. Superposition of ST1710 with related MarR family proteins. (A) ST1710, 1JGS, 1LJ9, 1LNW, 1S3J, 1UB9 and 1XMA are colored in red, green, blue, yellow, majenta, cyan and orange, respectively. (B) ST1710, 1Z91, 2A61, 2BV6, 2ETH and 2FBH are colored in red, green blue, yellow, majenta and cyan, respectively. (C) ST1710, 2FBI, 2FNP, 2HR3, 2NNN and 2NYX are colored in red, green blue, yellow, majenta and cyan, respectively. (D) ST1710, 2PEX, 2QWW, 3BDD, 3BJ6 and 3BJA are colored in red, green blue, yellow, majenta and cyan, respectively. (E) ST1710, 3BPV, 3BPX, 3BRO, 3CDH and 3CJN are colored in red, green blue, yellow, majenta and cyan, respectively. (F) ST1710, 3DEU, 3E6M, 3ECO, 3F3X and 3FM5 are colored in red, green blue, yellow, majenta and cyan, respectively. (G) ST1710, 3G3Z, 3HRM, 3JW4, 3KOL and 3KP6 are colored in red, green blue, yellow, majenta and cyan, respectively. (H) ST1710, 3NQO, 3NRV, 3OOP, 3Q5F and 3S2W are colored in red, green blue, yellow, majenta and cyan, respectively.

A


1 10 20 30 40 50 60 70 80 90 100
3GF2 MLESNENRIQIMSTIAKIYRAMSRELNRRLGEL-NLSYLDFLVLRATS--DGPKTMAYLANRYFVTQSAITASVDKLEEMGLVV
3F3X MQKIDEKLQLMNTIAKIYRGSIKEFNNRLGKLMNLSYLDFSILKATS--EEPRSMVYLANRYFVTQSAITAAVDKLEAKGLVR
2ETH MGSDKIHHHHHHMDALEIFKTLFSLVMRFSSYLPSMEEISDMKTTELYAFLYVAL--FGPKKMKEIAEFLSTTKSVNTNVVDSLEKRGLVV
3NQO GMDYSNELKELFLMNQTYATLFTLTNKIQIEGDKYFG--ILTSRQYMTILSILHLPEEETTLNNIARKMGTSKQNINRLVANLEKNGYVD
3NRV SNAMQKINIDRHATAQINNLANKLMLKSSTAYTQKFGIGMTEWRIISVLS-SASDCSVQKISDILGLDKRAVSRTVKKLEEKKYIE
1LJ9 TDILREIGHIARALDSISNIEFKELSLTRGQYLYLVRV-C-ENPGIIQEKIAELIKVDRTTAARAIKRLEEQGFIY
3BPV IPLKGLLSTILRSHRVFIGRELGHLNLTDAQVACLLRI-H-REPGIKQDELATFFHVDKGTIARTLRRLEESGFIE
3BPX GSHMDR---DIPLKGLLSTILRSHRVFIGRELGHLNLTDAQVACLLRI-H-REPGIKQDELATFFHVDKGTIARTLRRLEESGFIE
3S2W SNAMNTTEFDGISHAEGLCD---KEFIGKAISYLYRYGQIYIGKKIEPYGIGSGQFPFLMRL-Y-REDGINQESLSDYLKIDKGTTARAIQKLVDEGYVF
2FBH GHMAQTD--KHYFGTLLAQTSRAWRAELDRRLSHLGLSQARWLYLLHLAR-HRDSPTQRELAQSVGVEGPTLARLLDGLESQGLVR
3DEU MGHHHHHHHHHHSSGHIEGRHMLESPLGSDLARLVRIARALIDHRLKPLELTQTHWVTLHNIHQ-LPPDQSQIQLAKAIGIEQPSLVRTLDQLEDKGLIS
3Q5F SNAMESPLGSDLARLVRIARALIDHRLKPLELTQTHWVTLHNIHQ-LPPDQSQIQLAKAIGIEQPSLVRTLDQLEDKGLIS
3FM5 GHMAESQALSDDIGFLLSRVGGMVLGAVNKALVPTGLRVRSYSVLVLACE-QAEGVNQRGVAATMGLDPSQTVGLVDELEERGLVV
3OOP SNAMRGYYDEISFDVNTTAKKMHLFLMRSIASYDVTPEQWSVLEGI-E-ANEPISQKEIALWTKKDTPTVNRIVDVLLRKELIV
3CDH SNAMNDTPDDTFVSGYLLYLLAASSEERSAQFHDHIRAQGLRVPEWRVLACL-Y-DNDAMMITRLAKLSLMEQSRMTRIVDQMDARGLVT
3CJN SNAMAESTDQTEQLRELAEIG-LEGYAPYLMNRIMGRYNANLRKEMTALGLSTAKMRALAIL-S-AKDGLPIGTLGIFAVVEQSTLSRALDGLQADGLVR
3E6M SNAMTEARKIPKPSFPYGSPGELNSFLPYLLTRITHIWSSELNQALASEKLPTPKLRLLSSL-S-AYGELTVGQLATLGVMEQSTTSRTVDQLYDEGLAA
3K0L SNAMLRSSSVDRKREEEPR-------LSYMIARVDRIISKYLTEHLSALEISLPQFTALSVL-A-AKPNLSNAKLAERSFIKPQSANKILQDLLANGWIE
3BRO SNAMSRDLGRLLKIASNQMSTRFDIFAKKYDLTGTQMTIIDYLSRNKNKEVLQRDLESEFSIKSSTATVLLQRMEIKKLLY
3ECO MEFTYSYLFRHISHEMKQKADQKLEQFDITNEQGHTLGYLYAHQQDGLTQNDIAKALQRTGPTVSNLLRNLERKKLIY
3JW4 SNAMKESNHLMDTPYSYLIRSIGMKLKTSADARLAELGLNSQQGRMIGYIYENQESGIIQKDLAQFFGRRGASITSMLQGLEKKGYIE
2HR3 MPTNQDLQLAAHLRSQVTTLTRRLRREAQADPVQFSQLVYLGAIDR-LGGDVTPSELAAAERMRSSNLAALLRELERGGLIV
3BJ6 SNAMTHETDQLYQAVQATRPLLRNITAAVERGTLREGVTVGQRAILEGLSL-TPGATAP-QLGAALQMKRQYISRILQEVQRAGLIE
2NNN MSRTTPYRLDDQIGFILR-QANQRYAALFANGIGNGLTPTQWAALVRLGE-T-GPCPQNQLGRLTAMDAATIKGVVERLDKRGLIQ
3G3Z GPMNQLDQLGTRINLICNVFDKWIGQQDLNYNLFAVLYTLAT--EGSRTQKHIGEKWSLPKQTVSGVCKTLAGQGLIE
1LNW MNYPVNPDLMPALMAVFQHVRTRIQSELDCQ--RLDLTPDVHVLKLIDE--QRGLNLQDLGRQMCRDKRLITRKIRELEGRNLVR
1S3J MKSADQLMSDIQLSLQALFQKIQPEMLESMEKQ----GVTPAQLFVLASLKK--HGSLKVSEIAERMEVKPSAVTLMADRLEQKNLIA
2NYX MMPTEYPATAEESVDVITDALLTASRLLYRISAHSIAQVD-E----NITIPQFRTLVILSN--HGPINLATLLGVQPSATGRMVDRLVGAELID
2A61 GSMKQPFERILREICFNVKVEGRKVLRDF----GITPAQFDILQKIYF--EGPKRPGELSVLLGVAKSTVTGLVKRLEADGYLT
3BJA GMNNRELYGNIRDVYHLLQKNLDKAIEQY----DISYVQFGVIQVLAK--SGKVSMSKLIENNGCVPSNMTTMIQRMKRDGYVM
2FBI GHMSTPRPSLTLTLLQARERAMSFFRPSLNQH----GLTEQQWRVIRILRQ--QGEMESYQLANQACILRPSMTGVLARLERDGIVR
2QWW GMVGINTDTENISELLKTYWSIQRISAGYADQNAASLGLTIQQLAMINVIYS--TPGISVADLTKRLIITGSSAAANVDGLISLGLVV
3KP6 MVRRIEDHISFLEKFINDVNTLTAKLLKDLQTEYGISAEQSHVLNML-S---IEALTVGQITEKQGVNKAAVSRRVKKLLNAELVK
1JGS LFNEIIPLGRLIHMVNQKKDRLLNEY----LSPLDITAAQFKVLCSIRC--AACITPVELKKVLSVDLGALTRMLDRLVCKGWVE
3BDD GMQEMEDLLYRLKVADETISNLF----EKQLGISLTRYSILQTLLK--DAPLHQLALQERLQIDRAAVTRHLKLLEESGYII
1Z91 MENKFDHMKLENQLSFLLYASSREMTKQYKP-LLDKLNITYPQYLALLLLWE--HETLTVKKMGEQLYLDSGTLTPMLKRMEQQGLIT
2PEX MDTTTATTARTDTLLQLDNQLSFALYSANLAMHKLYRG-LLKALDLTYPQYLVMLVLWE--TDERSVSEIGERLYLDSATLTPLLKRLQAAGLVT
2BV6 GSHMNLKEQLCFSLYNAQRQVNRYYSNKVFKKYNLTYPQFLVLTILWD--ESPVNVKKVVTELALDTGTVSPLLKRMEQVDLIK
3HRM GSHMYLSKQLCFLFYVSSKEIIKKYTN-YLKEYDLTYTGYIVLMAIEN--DEKLNIKKLGERVFLDSGTLTPLLKKLEKKDYVV
2FNP MAITKINDCFELLSMVTYADKLKSLIKKEFSISFEEFAVLTYISENKEKEYYFKDIINNLNYKQPQVVKAVKILSQEDYFD
1XMA MGSSHHHHHHSSGLVPRGSQSTSLYKKAGLMVISSDVIRGYVDTIILSLLIEGDSYGYEI-------SKNIRIKTDELYYIKETTLYSAFARLEKNGYIK
1UB9 MEELKEIMKSHILGNPVRLGIMIFLLPRRKAPFSQIQKVLDLTPGNLDSHIRVLERNGLVK
Consensus .....q...l.....l.....$e..gl..

101 110 120 130 140 150 160 170 180
3GF2 RVRD--REDRRKILIEITEKGLETFNKGIEIYKKLANEVTGDLSEDEVILVLDKISKILKRIEEISQ
3F3X RIRD--SKDRRIVIVEITPKGRQVLLEANEVLRNLVNEMLSDVENVEELL--EGLNKILSRIGSSKD
2ETH REMD--PVDRRTYRVVLTEKGKEIFGEILSNFESLLKSVLEKFSEEDFKVVSEGFNRMVEALSREGR
3NQO VIPS--PHDKRAINVKVTDLGKKVMVTCSRTGINFMADVFHEFTKDELETLWSLLKKMYRFNGEEQDGFEEDAN
3NRV VNGH--SEDKRTYRINLTEMGQELYEVASDFAIEREKQLLEEFEEAEKDQLFILLKKLRNKVDQM
1LJ9 RQED--ASNKKIKRIYATEKGKNVYPIIVRENQHSNQVALQGLSEVEISQLADYLVRMRKNVSEDWEFVKKG
3BPV REQD--PENRRRYILEVTRRGEEIIPLILKVEERWEDLLFRDFTEDERKLFRKMCRRLAEERVRMR
3BPX REQD--PENRRRYILEVTRRGEEIIPLILKVEERWEDLLFRDFTEDERKLFRKMCRRLAEERVRMRGEW
3S2W RQRD--EKDRRSYRVFLTEKGKKLEPDMKKIASEWGEILFSSFDDRQRREITNSLEIMFENGLKIM
2FBH RLAV--AEDRRAKHIVLTPKADVLIADIEAIAASVRNDVLTGIDESEQALCQQVLLRILANLENR
3DEU RQTC--ASDRRAKRIKLTEKAEPLIAEMEEVIHKTRGEILAGISSEEIELLIKLIAKLEHNIMELHSHD
3Q5F RQTC--ASDRRAKRIKLTEKAEPLIAEMEEVIHKTRGEILAGISSEEIELLIKLIAKLEHNIMELHSHD
3FM5 RTLD--PSDRRNKLIAATEEGRRLRDDAKARVDAAHGRYFEGIPDTVVNQMRDTLQSIAFPTFVEGS
3OOP REIS--TEDRRISLLSLTDKGRKETTELRDIVEASCEKMFAGVTRTDLEQFTAILKNISTNIE
3CDH RVAD--AKDKRRVRVRLTDDGRALAESLVASARAHETRLLSALADTDAARIKGVLRTLLDVLDRPRESR
3CJN REVD--SDDQRSSRVYLTPAGRAVYDRLWPHMRASHDRMFQGITPQERQAFLATLNKMLANIRVHEI
3E6M RSIS--DADQRKRTVVLTRKGKKLAETSPLINDFHAELVGNVDPDKLQTCIEVLGEILKGKTDY
3K0L KAPD--PTHGRRILVTVTPSGLDKLNQCNQVVQQLEAQMLQGVDINLAFLIRNNLELMVKNLSTFSSLDQSKE
3BRO RKVS--GKDSRQKCLKKLTKKANKLETIILSYMDSDQSQMTSGLNKEEVVFLEKILKRMIESD
3ECO RYVD--AQDTRRKNIGLTTSGIKLVEAFTSIFDEMEQTLVSQLSEEENEQMKANLTKMLSSLQ
3JW4 RRIP--ENNARQKNIYVLPKGAALVEEFNNIFLEVEESITKGLTKDEQKQLMSILIKVNRSM
2HR3 RHAD--PQDGRRTRVSLSSEGRRNLYGNRAKREEWLVRAMHACLDESERALLAAAGPLLTRLAQFEEP
3BJ6 RRTN--PEHARSHRYWLTPRGEAIITAIRADEMAKLALFSEGFSSVELTAYHKVQLALTRFFADLAKEA
2NNN RSAD--PDDGRRLLVSLSPAGRAELEAGLAAAREINRQALAPLSLQEQETLRGLLARLI
3G3Z WQEG--EQDRRKRLLSLTETGKAYAAPLTESAQEFSDKVFATFGDKRTTRLFADLDALAEVMEKTISENKK
1LNW RERN--PSDQRSFQLFLTDEGLAIHQHAEAIMSRVHDELFAPLTPVEQATLVHLLDQCLAAQPLEDI
1S3J RTHN--TKDRRVIDLSLTDEGDIKFEEVLAGRKAIMARYLSFLTEEEMLQAAH-ITAKLAQAAETDEKQNMKRGNG
2NYX RLPH--PTSRRELLAALTKRGRDVVRQVTEHRRTEIARIVEQMAPAERHGLVRALTAFTEAGGEPDARYEIERSHHHHHH
2A61 RTPD--PADRRAYFLVITRKGEEVIEKVIERRENFIEKITSDLGKEKSSKILDYLKELKGVMERNFSKQ
3BJA TEKN--PNDQRETLVYLTKKGEETKKQVDVQYSDFLKENCGCFTKEEEGILEDLLLKWKKHLN
2FBI RWKA--PKDQRRVYVNLTEKGQQCFVSMSGDMEKMYQRIQERFGEEKLAQLLELLNELKKIKP
2QWW KLNKTIPNDSMDLTLKLSKKGED-LSKRSTANAFMYKAMMKVFENLTENEIEELIRL-NKKVETLLKKSK
3KP6 LEKPDSNTDQRLKIIKLSNKGKKYIKERKAIMSHIASDMTSDFDSKEIEKVRQVLEIIDYRIQSYTSKL
1JGS RLPN--PNDKRGVLVKLTTGGAAIC----EQCHQLVGQ--DLHQELTKNLTADEVATLEYLLKKVLP
3BDD RKRN--PDNQREVLVWPTEQAREALITNPSAHHQAIKT--SMNQILTVEESEQFLATLDKLLIGLQNLPI
1Z91 RKRS--EEDERSVLISLTEDGALLKEKAVDIPGTILGL--SKQSGEDLKQLKSALYTLLETLHQKN
2PEX RTRA--ASDERQVIIALTETGRALRSKAGAVPEQVFCR--SACSLDELRQLKQELEKLRSSLGAG
2BV6 RERS--EVDQREVFIHLTDKSETIRPELSNASDKVASA--SSLSQDEVKELNRLLGKVIHAFDE
3HRM RTRE--EKDERNLQISLTEQGKAIKSPLAEISVKVFNE--FNISERERASDIINNLRNFVSKNF
2FNP KKRN--EHDERTVLILVNAQQRKKIESLLSRVNKRITEANNEIEL
1XMA SYYGEETQGKRRTYYRITPEGIKYYKQKCEEWELTKKVINKFVKELESNGDN
1UB9 TYKV--IADRPRTVVEITDFGMEEAKRFLSSLKAVIDGLDL
Consensus r... ..d.R...lt..g.....e.....l.....

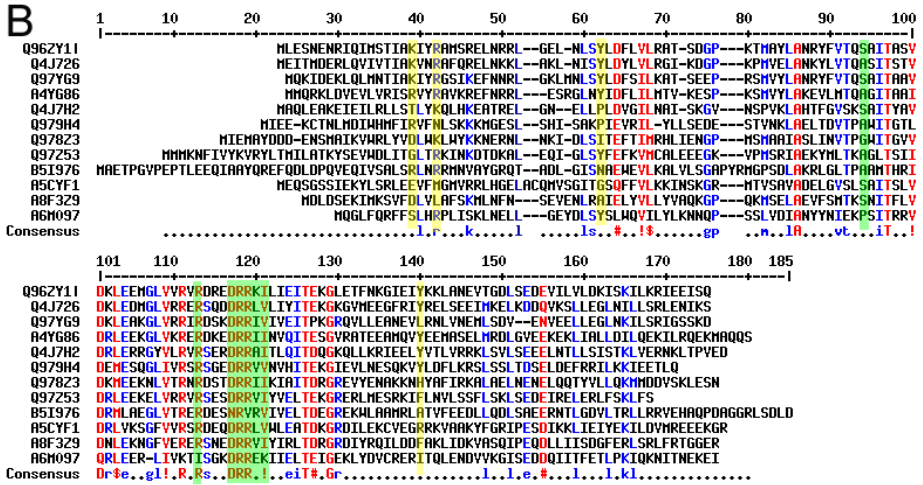


Fig. 3. Sequence alignment of ST1710 and its structurally and sequenctially related proteins from different species. (A) Structurally related proteins to ST1710, based on the Dali Zscore. (B) Sequentially related proteins from the non-reduntant sequence database. The highly redundant proteins are removed. The ligand and DNA binding residues are highlighted with yellow and green shades, respectively.

Metallosphaera sedula, Thermoplasma acidophilum, Thermoplasma volcanium, Streptomyces sviceus, Pelotomaculum thermopropionicum, Thermotoga lettingae, Clostridium beijerinckii and others. Among these, the amino acid sequence of ST1710 displays about 50% identity to the *S. acidocaldarius* (Chen et al., 2005) (17) and *S. solfataricus* (She et al., 2001) sequences, 41% identity to the M. sedula sequence (Copeland et al., 2006) and approximately 30-40% identity to others (Fig. 3).

2.1.1 Interactions between MarR homologues and ligands

MarR homologues are known to bind a variety of lipophilic compounds, including salicylate, ethidium and CCCP (Table 1). These bound molecules control interaction between protein-DNA molecules. Sodium salicylate is a well-known example of a compound that can inhibit MarR activity both *in vitro* and *in vivo* at millimolar concentrations (Alekshun and Levy, 1999b). Three different MarR proteins have been solved with the salicylate ligand, including ST1710 from *S. tokodaii*, MarR from *E. coli* and MTH313 from *Methanobacterium thermoautotrophicum*. Among these, ST1710 is the only MarR homologue solved in the apo form, complexed with salicylate ligand and complexed with a putative promoter DNA (Kumarevel et al., 2009). One salicylate ligand is identified and located at the interface between the helical dimerization and wHtH DNA-binding domains in ST1710 (Fig. 1A&B), and the bound salicylate ligand shows many interactions with the surrounding protein residues. In particular, the O2′ of salicylate is bonded to the side chain oxygens of Tyr37 and Tyr111. In addition, the side chain oxygen of Tyr37 is also bonded to the O1′ of the salicylate ligand molecule. The ligand oxygen O1′ is hydrogen bonded to the amino group (NH2) of residue Arg20, while the O2 of the ligand molecule is hydrogen bonded to the side chain nitrogen of

Lys17. The latter two interactions are from the symmetrically related molecule. Notably, all of the ST1710 residues that interact with the ligand are highly conserved among closely related species (>40% identity) (Fig. 3).

In contrast to ST1710, *E. coli* MarR was solved with two salicylate molecules per dimer, and both of them are highly exposed to the solvent. These salicylate binding sites are also not comparable to that of ST1710. The bound salicylate is hydrogen bonded with some of the MarR residues (Ala70, Thr72, Arg77, Arg86); however, the physiological relevance of either salicylate binding site could not be determined (Fig. 4). It seems that salicylate may stabilize the crystal packing, since in its absence, the crystals cannot be used for structure determination in the case of *E. coli* MarR (Alekshun et al., 2001). Analyses of another MarR homolog from *M. thermoautotrophicum* MTH313, which was also solved in the free (apo) form and complexed with salicylate, revealed a large asymmetrical conformational change that is mediated by the binding of sodium salicylate to two distinct locations in the dimer (Saridakis et al., 2008) (Fig. 4). The bound salicylate has two direct and one water mediated interactions with MTH313. Although the ligand binding sites in ST1710 and MTH313 are comparable, we have not found any conformational changes in ST1710 between the apo and ligand bound complexes, as observed in MTH313.

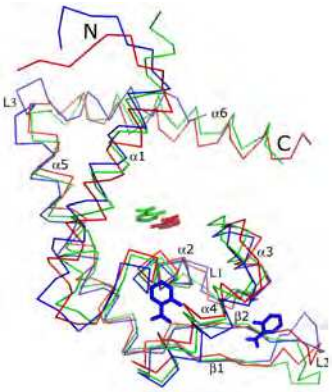


Fig. 4. Salicylate binding analysis in MarR homologues. Superposition of the ST1710-salicylate complex with other known MarR family of protein crystallized in the presence of salicylate. The ST1710, *E. coli* MarR and *M. thermoautotrophicum* MTH313 are shown in green, blue and red, respectively.

Meanwhile, eight salicylate molecules are bound to *Staphylococcus epidermidi*s of TcaR (Chang et al., 2010). Among these eight molecules, two are bound similarly to that with MTH313, while the other two were observed in the more shallow binding pocket in each monomer. The remaining ligands are highly exposed to the solvent. TcaR has also been crystallized with four different antibiotics (ampicillin, kanamycin, methicillin and penicillin), revealing their interactions with the protein (Chang et al., 2010). The available biochemical and biophysical results suggest that the MarR regulators modulate the DNA binding affinity in the presence of ligands or drug molecules. However, more ligand bound complexes are required to generalize the binding pocket properties as well as to understand how these MarR regulators allosterically change their conformation in the presence of vaious drugs/ligands to mediate the protein-DNA interactions.

Protein	Organism	Footprint (DNA)	K_d (nM)	Ligand	K_d	Reference
ST1710	*Sulfolobus tokodaii*	30	200 ~ 1500	Salicylate, Ethidium Bromide, CCCP	~2-25 μM	Kumarevel et al., 2008 & 2009 Yu et al., 2009
MepR	*Staphylococcus aureus*	27,44	6.3	Ethidium, DAPI, Rhodamine 6G	~3-63 μM	Kumaraswami et al., 2009
MarR	*Escherichia coli*	21	1	Salicylate, Plumbagin, 2,4-dinitrophenol, menadione	0.5 – 1 mM	Martin & Rosner 1995; Cohen et al., 1993b; Seoane & levy, 1995; Alekshun & Levy 1999b; Alekshun et al, 2001
EmrR	*Escherichia coli*	42	-	Salicylate, Caronyl cyanide m-chlorophenylhydrozone, 2,4,-dinitrophenol, ethidium bromide	1.3 -11.1 μM	Xiong et al. 2000; Brooun et al., 1999
MexR	*Pseudomonos aeruginosa*	28	-	β-lactamin		Evans et al., 2001.
CbaR	*Comamonas testosteroni*	22	-	3-chlorobenzoate, protocatechuate		Providenti & Wyndham, 2001
CinR	*Butyrivibro fibrisolvens*	-	-	Cinnamic acid sugar esters		Dalrymple & Swadling, 1997
HpaR	*Escherichia coli*	27	-	4-hydroxyphenyl-acetic acid, 3-hydroxyphenyl acetic acid, 3,4-hydroxyphenyl acetic acid		Galan et al., 2003
ExpG	*Sinorhizobium meliloti*	21	0.58-1.3			Bartels et al, 2003; Baumgarth et al., 2005
PecS	*Erwinia chrysanthemi*	45	4-200			Reverchon et al., 2002; Rouanet et al., 2004
SlyA	*Salmonella typhimurium*	25	-			Stapleton et al., 2002
OhrR	*Xanthomonas campestris*	44	-	Tert-butyl hydroperoxide, cumene hydroperoxide		Mongkolsuk et al., 2002
OhrR	*Bacillus subtilis*	42	5	Tert-butyl hydroperoxide, cumene hydroperoxide		Fuangthong et al., 2001; Fuangthong & Helmann, 2002; Panamanee et al., 2002
HucR	*Deinococcus radiodurans*	21	0.29	Uric acid, Salicylate	11.6 μM	Wilkinson & Grove, 2004; 2005

Table 1. DNA and ligand binding data for MarR homologues.

2.1.1 Interactions between MarR proteins and DNA

It is well-known that members of the MarR family of regulatory proteins bind to their cognate double-stranded DNA by their winged HtH motif (Alekshun et al., 2001; Hong et al., 2005; Kumarevel et al., 2009). Footprinting analyses suggested that different MarR regulators recognize promoters of different lengths with different affinities (Table 1). In an earlier study, we have used the *OhrR* promoter sequence as a search model to identify the putative promoter DNA sequence for ST1710 from the *S. tokodaii* genomic sequence (Kumarevel et al., 1998). We have also shown the binding constant for DNA to be around 15 μM using gel mobility shift assays. Yu et al. (2009) subsequently showed by fluorescence spectroscopy that the affinity of the same DNA promoter we identified is increased significantly with increasing temperature. The affinity was shown to be approximately double from 10°C (K_d = 618 ± 34 nM) to 30°C (K_d = 334 ± 15 nM) and from 30°C to 50°C (K_d = 189 ± 9 nM). We later crystallized ST1710 along with two different DNA promoters (30-mer and 26-mer) and revealed the protein-DNA interactions and mode of binding as summarized below (Kumarevel et al., 2009).

The overall structure of the ST1710-DNA complex is shown in Fig. 5A & B. The bound DNA adopts a B-form right-handed structure, passing over the protein molecule by only contacting at the winged HtH loop regions. The wHtH domains recognize the promoter DNA (TAACAAT) (15-21) region, consistent with the -10 region of the OhrR-*ohrA* operator complex. The 4 and 3 bases at the 5′ and 3′-ends are highly disordered and hence not modeled. Of the bound 46 nucleotides, only 22 nucleotides were found to be involved in 36 contacts with six protein molecules. The critical protein-DNA contacts observed in this complex are as follows: Ser65 - Thy5′; Arg84 - G13′ and Ade17; Arg89 - Thy14′; Arg90 - Cyt18; Asp88 – Cyt18 (two salt bridge contacts); Lys91 - Ade19; Ile91 - Ade20. The observed salt bridge may be important in fixing the conformation of residue Arg90 in order to make contact with the nucleic acid base, Cyt18. Thus, the following residues Ser65, Arg84, Asp88, Arg89, Arg90, Lys91 and Ile92 interact with the bound promoter DNA. As further clarification of these protein-DNA interactions, our analysis of three mutant proteins (Arg89Ala, Arg90Ala, Lys91Ala) at the DNA binding loop region in gel mobility shift assays clearly support that these positively charged residues are important for DNA binding (Kumarevel et al., 2009). The DNA-binding residues in ST1710 are highly conserved among the closely related proteins Fig. (3). The winged loop region connecting the strands β1 and β2 apparently plays a major role in modulating their conformation for binding to the DNA molecule, and this mode of recognition is anticipated for the proteins closely related to ST1710 as well as those in the family of MarR regulators.

In our earlier report, we noticed only a small difference at the loop region connecting strands β1 and β2 in the protein conformers crystallized in two different space groups, but the overall structures are otherwise identical (Kumarevel et al., 2008). Similarly, we have not observe any conformational changes in comparisons of the ST1710-salicylate complex and native structure crystallized under the same conditions, and the subunits in the dimer are identical. In contrast to these observations, a significant conformational change has been observed between subunits (A, B chains) in the ST1710-DNA complex, although the overall structural topology remains identical. Specifically, the C-terminal helix and the winged HtH motif region show displacement relative to the other. The DNA binding motif is elevated

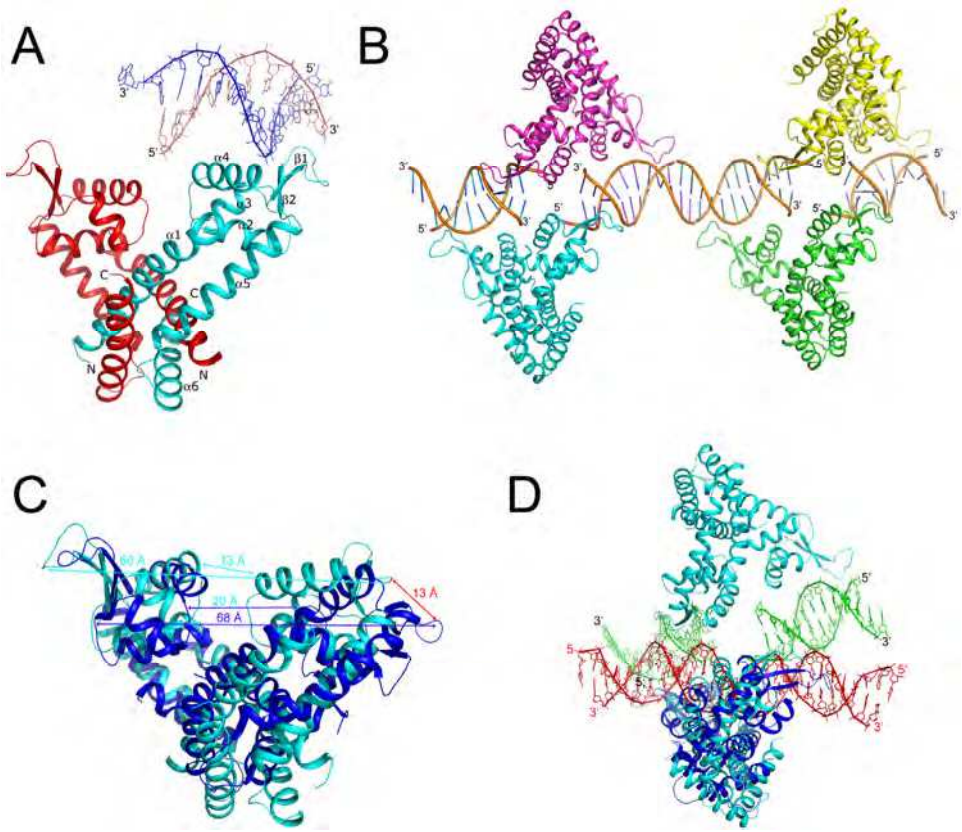


Fig. 5. Structure of ST1710-DNA complex and it's structural comparison with OhrR-*OhrA* complex. (A) ST1710-DNA complex observed in the aymmetric unit. The secondary structural assignments, N-/C- termini ends are labeled in one of the dimeric monomers. The complexed nucleic acids are shown as stick representations. (B) Part of the packing diagram. The 5'- and 3'- ends of each nucleotide chain is labeled. (C, D) Superposition of the OhrR-*OhrA* complex on the ST1710-DNA complex is shown without (A) and with nucleic acids (B). The protein and nucleic acids are shown in St1710-DNA complex are in cyan and green; while those in OhrR-*OhrA* complex are shown in blue and red, respectively.

compared to the other chain, while the C-terminal helix α6 is lower down. It is noteworthy to mention that the distances between the wHtH domains in the dimer are reduced by ~10 Å for the ST1710-DNA complex, compared to the native and salicylate complexes. These observed conformational changes are required in order to facilitate the DNA-binding and thus would explain the conformational flexibility of MarR homologues.

Another member of the MarR family of regulators that has been solved in complex with a promoter sequence is the *B. subtilis* OhrR. The OhrR was crystallized in the presence of a 29-mer duplex containing the -10 region of the cognate DNA. In the OhrR-*OhrA* complex, the wHtH motif contacts the DNA promoter sequence with substantial widening and deepening

of the major groove that results from insertion of the recognition helix (α4) of the wHtH motif. The wHtH and recognition helices make many contacts with the DNA directly or mediated through water. The wHtH domain is important for the DNA interaction as evidenced by several mutagenic analyses, which show that the positively charged residues (Arg94) located at the terminals are important for the DNA contacts in *E. coli* MarR. In the OhrR-*ohrA* complex, the distance between wHtH loops is around 67 Å, and the distance between the recognition helices (α4) is about 20 Å, although the wings of the subunits are translocated about 16 Å compared to the structure of reduced OhrR (Hong et al., 2005) (Fig. 5C). In an attempt to clarify the binding mechanism of MarR regulators, a comparative analysis of our ST1710-DNA complex with the OhrR-*ohrA* complex (Fig. 5C & D) was performed, which revealed large conformational changes between these two complexes. Interestingly, we also observed unique conformational changes in the mode of DNA recognition. In contrast to the OhrR-*OhrA* complex, the bound promoter DNA passed over the wHTH motif without deepening the structure through the 2-fold axis in the ST1710-DNA complex. Despite their differences, it is interesting to note that the protein contacting residues are highly conserved between these two proteins and among the MarR family of regulators. This unexpected mode of DNA-binding in ST1710 is caused by one of the subunits translocated around 13 Å towards the 2-fold axis, reducing the distance between the recognition helix of the subunits to 13 Å. Thus, the mode of DNA binding observed in the OhrR-*ohrA* operator complex would be impossible for that of ST1710. Such unique conformational changes observed in these complexes explain how the MarR homolog regulators can modulate the DNA-binding affinity based on the cognate promoter or ligand molecules.

3. Conclusion

The MarR family of regulatory proteins in bacteria and archaea regulate a variety of biological functions, including those associated with the development of antibiotic resistance, a growing global health problem. Based on the existing crystal structures, it seems that members of the MarR family of proteins adopt similar topology, despite variations in sequence similarities among them. We have solved the crystal structure of ST1710 in three different forms (apo-form, ST1710-salicylate and ST1710-DNA complex) and demonstrated the functional importance of the ligand binding and DNA binding residues. The ligand or drug binding to the MarR regulators may regulate their promoter binding abilities as evidenced with MarR, ST1710 and MTH313. Furthermore, the promoter DNA is also recognized by the protein in a unique fashion as observed in OhrR-*OhrA* and ST1710-DNA complexes. Taken altogether, the current evidence describe the MarR regulators containing wHTH motifs as being prone to binding DNA through their positively charged residues located in their loops, and the mode of DNA binding depends on the subunit organization as observed in the MarR family of proteins (ST1710, OhrR). Through further structural and functional studies on MarR-DNA binding, we will be better poised to develop new drugs to specifically target those interactions that confer drug resistance to pathogenic organisms.

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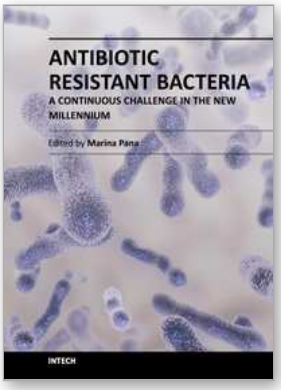

Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium

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