Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Identification of disulphide stress–responsive extracytoplasmic function sigma factors in *Rothia mucilaginosa*

Takayuki Nambu [a,*], *Kazuyoshi Yamane* [a], *Takeshi Yamanaka* [a], *Chiho Mashimo* [a], *Hugo Maruyama* [a], *Masahiro Yoshida* [b], *Hiroyuki Hayashi* [b], *Kai-Poon Leung* [c], *Hisanori Fukushima* [a]

[a] *Department of Bacteriology, Osaka Dental University, 8-1 Kuzuha-Hanazono, Hirakata, 573-1121 Japan*
[b] *Department of Endodontics, Osaka Dental University, 8-1 Kuzuha-Hanazono, Hirakata, 573-1121 Japan*
[c] *US Army Denal and Trauma Research Detachment, Institute of Surgical Research, 3650 Chambers Pass, Fort Sam Houston, TX 78234-6315, USA*

ARTICLE INFO

Article history:
Accepted 23 October 2012

Keywords:
Rothia mucilaginosa
ECF sigma factor
Disulphide regulation

SUMMARY

Rothia mucilaginosa is known as a member of commensal bacterial flora in the oral cavity and has received attention as a potential opportunistic pathogen. We previously determined the genomic sequence of *R. mucilaginosa* DY-18, a clinical strain with biofilm-like structures isolated from an infected root canal of a tooth with persistent apical periodontitis. We found that the DY-18 genome had only two sigma factor genes that encoded the primary and extracytoplasmic function (ECF) sigma factors. Genomic analysis on the available database of *R. mucilaginosa* ATCC 25296 (a type strain for *R. mucilaginosa*) revealed that ATCC 25296 has three sigma factors: one primary sigma factor and two ECF sigma factors, one of which was highly homologous to that of DY-18. ECF sigma factors play an important role in the response to environmental stress and to the production of virulence factors. Therefore, we first examined gene-encoding sigma factors on *R. mucilaginosa* genome *in silico*. The homologous ECF sigma factors found in strains DY-18 and ATCC 25296 formed a distinct SigH (SigR) clade in a phylogenetic tree and their cognate anti-sigma factor has a HXXXCXXC motif known to respond against disulphide stress. Quantitative reverse transcription polymerase chain reaction (PCR) and microarray analysis showed that the transcriptional levels of *sigH* were markedly up-regulated under disulphide stress in both strains. Microarray data also demonstrated that several oxidative-stress-related genes (thioredoxin, mycothione reductase, reductase and oxidoreductase) were significantly up-regulated under the diamide stress. On the basis of these results, we conclude that the alternative sigma factor SigH of *R. mucilaginosa* is a candidate regulator in the redox state.

© 2012 Elsevier Ltd. All rights reserved.

* *Corresponding author*. Tel.: +81 72 864 3051; fax: +81 72 864 3151.
E-mail address: nambu-t@cc.osaka-dent.ac.jp (T. Nambu).

0003–9969/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.archoralbio.2012.10.017

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JUN 2013	**N/A**	**-**

4. TITLE AND SUBTITLE	
Identification of disulphide stressâresponsive extracytoplasmic function sigma factors in Rothia mucilaginosa.	5a. CONTRACT NUMBER
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Nambu T., Yamane K., Yamanaka T., Mashimo C., Maruyama H., Yoshida M., Hayashi H., Leung K. P., Fukushima H.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**9**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

1. Introduction

Rothia mucilaginosa, formerly classified as *Stomatococcus mucilaginosus*, is a Gram-positive, coagulase-negative, non-spore-forming coccus with relatively high guanine–cytosine (GC) content of deoxyribonucleic acid (DNA).[1] This organism belongs to the actinobacteria, which includes the genera *Actinomyces*, *Corynebacterium*, *Mycobacterium* and *Streptomyces*, and is known as a member of the commensal bacterial flora in the oral cavity and the upper respiratory tract.[1,2] Although several studies have suggested that this organism is a member of healthy microbiota,[3,4] there have been an increasing number of reports on infections caused by *R. mucilaginosa*, especially among immunocompromised patients.[5–10] More recently, Zamakhchari et al.[11] showed that *R. mucilaginosa* were highly effective in degrading gluten peptides harmful to coeliac disease patients (gluten-sensitive enteropathy).

In dentistry, *Rothia* species are also found in clinical samples taken from the infected root canals of teeth with apical periodontitis, and in some cases persist after repetitive endodontic treatment.[12–14] Thus, *R. mucilaginosa* seems to have potential to survive under harmful environmental conditions like other oral bacteria do;[15–19] however, it still remains unclear how *Rothia* species respond to environmental stress. The responsiveness and adaptation of a few actinobacteria to various environmental stresses are known to be principally regulated by sophisticated transcriptional systems, such as extracytoplasmic function (ECF) sigma factors or one- or two-component systems.[20,21]

We previously determined the genomic sequence of *R. mucilaginosa* DY-18, a clinical isolate from a persistent apical periodontitis lesion. The genome of DY-18 encodes two sigma factors, one of which is an ECF sigma factor.[14] Compared to other actinobacteria, the number of sigma factors in *R. mucilaginosa* is relatively small (microbial signal transduction (MiST2) database, http://mistdb.com/); however, the response of this organism against environmental stresses *via* sigma factors still remains to be elucidated.

In order to obtain a cue to study how the sigma factors in *R. mucilaginosa* play a role in environmental stress responses, we examined gene-encoding sigma factors on the genomes of *R. mucilaginosa* strains DY-18 and ATCC 25296, a type strain of *R. mucilaginosa*, by means of bioinformatics and gene expression analyses. Several lines of evidence obtained from this study suggested that the ECF sigma factor of *R. mucilaginosa* is a candidate regulator in a redox state. Microarray analysis and real-time reverse transcription polymerase chain reaction (RT-PCR) revealed that the transcriptional level of the ECF sigma factor genes was significantly up-regulated under disulphide stress.

2. Materials and methods

2.1. Bacterial strains and bioinformatics analysis

R. mucilaginosa strains DY-18[14] and ATCC 25296 were used in this study. The genome sequences of these strains are available at DNA Data Bank of Japan (DDBJ) (accession number, AP011540) and National Center for Biotechnology Information (NCBI) (accession number, ACVO01000001–ACVO01000025), respectively. Because we hypothesised that the sigma factors of *R. mucilaginosa* somehow relay the environmental stress signals to the gene expression necessary for responding to oxidative stress, we first examined profiles of these genes using bioinformatics analysis on available databases such as MiST2 (http://mistdb.com/)[22] and Basic Local Alignment Search Tool (BLAST).[23] Amino acid sequences of actinobacterial ECF sigma and anti-sigma factors were obtained from MiST2 and the NCBI (GenBank) protein sequence databases. Sequences were aligned using multiple alignment with fast Fourier transform (MAFFT, v6.851b) with strategy L-INS-I.[24] Phylogenetic analyses were performed with the Neighbor-joining option in MAFFT using all ungapped sites and with 1000 bootstrapping resamplings. A phylogenetic tree and sequence alignment were drawn with Archaeopteryx 0.957 (http://www.phylosoft.org/archaeopteryx/) and Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html), respectively. A highly homologous group of sigma factors was identified by a BLASTP search of GenBank with the amino acid sequence of ROTMU0001_0778 in ATCC 25296.[23,25]

2.2. Diamide treatment and ribonucleic acid (RNA) isolation

Because bioinformatics analysis of *R. mucilaginosa* genomes suggested that the tandem genes encoding an ECF sigma factor and an anti-sigma factor are involved in a regulation of disulphide control (see below), we next examined transcriptional levels of these genes under diamide treatment described elesewhere.[26] Each strain was grown aerobically at 37 °C in trypticase soy broth (TSB) supplemented with 5% yeast extract (TSB-Y) with shaking or on TSB-Y agar (Becton Dickinson, Franklin Lakes, NJ, USA). To investigate the transcriptional levels of sigma factor genes, we grew each strain in TSB-Y to an optical density at 600 nm of 0.4 and divided each one into two 10-ml aliquots. Diamide (Sigma–Aldrich, St. Louis, MO, USA) was added to one of the aliquots to a final concentration of 5 mM. After 30-min incubation at 37 °C with shaking, bacteria were chilled on ice. Cells were collected by centrifugation at 10,000 × *g* for 10 min at 4 °C. Total RNA was extracted from cells by using the RiboPure bacterial RNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's guidelines and was treated with DNase I (included in the kit) to remove any contaminating DNA. The concentration of isolated RNA samples was measured spectrophotometrically on the basis of absorbance at 260 nm (GeneQuant pro, GE Healthcare, Waukesha, WI, USA).

2.3. Quantitative real-time RT-PCR

The messenger RNA (mRNA) levels of targeted genes were examined by quantitative real-time RT-PCR (qRT-PCR). Genes and specific primers designed using *in silico* Molecular Cloning Genomics Edition (version 4.1, In Silico Biology, Inc., Yokohama, Japan) are listed in Table 1. qRT-PCR was performed with the MiniOpticon RT-PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the iScript one-step RT-PCR kit with SYBR green (Bio-Rad Japan, Tokyo, Japan)

Table 1 – Primers used in this study.

Primers	Sequences (5′–3′)
RT-PCR	
DY_sigA_RT_F	AGTGGAGCTGTTCCTGCAG
DY_sigA_RT_R	AGAACTCGGCAAGGAACTC
DY_sigH_RT_F	TGCAGGAAACCTACATGAAG
DY_sigH_RT_R	TGATGTACGTATTCGTCAGG
DY_rpoB_RT_F	ACATACGACCGTAGTGCG
DY_rpoB_RT_R	TCGCAGTTCATGGACCAG
DY_gyrB_RT_F	TCTTCGGCTTCAAAGGAG
DY_gyrB_RT_R	GACGAGGTTGCTGAAGAG
ATCC_sigA_RT_F	CAAGGACTACCTCAAGCAG
ATCC_sigA_RT_R	TGAGGTCCTTGTCCATGTC
ATCC_sigH_RT_F	ACCGCATCCTGACGAATAC
ATCC_sigH_RT_R	CTCACGAACCTCCGTATTC
ATCC_sigRA_RT_F	TGATGCAGAAGGACTCGAAG
ATCC_sigRA_RT_R	ACTTCACTGGACTCAGCCG
ATCC_rpoB_RT_F	TGGACATCTACCGCAAGC
ATCC_rpoB_RT_R	CTGGCTCAGAACGGAAGC
ATCC_gyrB_RT_F	AGTCCGTCTACTCGTACGC
ATCC_gyrB_RT_R	GAGGTTATCGTCCTTATCG
Primer extension analysis	
PE_sigA_F	TTGCAGACAAAGCACCCCACCA-CAAGCCAG
PE_sigA_R	TGGTTGCCTTCTTGGTGCGGGTGG-TCTTGG
PE_sigH_F	ACGCCCGGCGCGTTCCTGTAGAG-GAGAAAC
PE_sigH_R	GCAGGGCGGCAGCGTAGAGCT-GATCCACAT

according to the manufacturer's protocols. Data were analysed with Bio-Rad CFX Manager Software, and expression levels of genes with or without diamide treatment were normalised to *gyrB* expression and were reported as a fold change.

2.4. *Primer extension analysis*

The transcriptional start sites were identified by a primer extension assay using a 6-carboxyfluorescein (FAM)-labelled primer and the GeneScan analysis system (Applied Biosystems Japan, Tokyo, Japan) as described by Lloyd et al.,[27] with some modifications. Purified RNA was reverse transcribed into complementary DNA (cDNA) with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) by using primer PE_sigA_R or PE_sigH_R (Table 1) to form FAM-labelled cDNA according to the manufacturer's protocols. cDNA samples were mixed with a GeneScan LIZ-500 internal size standard (Applied Biosystems) and denatured at 95 °C for 5 min. Then, samples were chilled quickly on ice and loaded onto an ABI PRISM 3130*xl* sequencer (Applied Biosystems). To accurately determine the reverse transcriptional product size of *sigH* or *sigA*, PCR reactions were performed with genomic DNA as the template. The products amplified with the primers used in the extension analysis and newly designed primers (PE_sigA_F or PE_sigH_F, Table 1) from the sequences around the determined transcription starting points were used as controls. Electrophoretograms were aligned according to the size standards using the GeneMapper v4.0 software (Applied Biosystems).

2.5. *Microarray analysis*

A whole genome microarray for *R. mucilaginosa* DY-18 was custom-designed and provided by NimbleGen Systems, Inc. (Madison, WI, USA). In this microarray, each of the 1992 coding sequences (CDS) in the genome of *R. mucilaginosa* DY-18 (GenBank accession number NC_013715) was represented by nine probes of 60-mer oligonucleotides, and each chip was designed to have four technical replicates. The cDNA synthesis, hybridisation and image acquisitions were performed by NimbleGen Systems, Inc. For each condition, two independent biological replicates containing a technical duplicate were tested. Data analysis was performed using Subio platform version 1.6 (Subio Inc., Tokyo, Japan).

3. Results

3.1. *In silico characterisation of the ECF sigma factors*

The genome of *R. mucilaginosa* DY-18 encodes two sigma factors: the essential sigma factor designated as SigA (RMDY18_07350) and the alternative sigma factor (RMDY18_07090), which belongs to the ECF subgroup.[14] According to the MiST2 database,[22] the draft genome of *R. mucilaginosa* ATCC 25296 encodes two ECF sigma factors (ROTMU0001_0778 and ROTMU0001_0903). To gain insight into the function of the ECF sigma factor in this species, a multiple alignment of the amino acid sequences of selected sigma factors was performed with the MAFFT program.[24] Then, the unrooted phylogenetic tree was obtained by using the neighbour-joining algorithm.[28] In this phylogenetic tree, two ECF sigma factors (RMDY18_07090 in DY-18 and ROTMU0001_0903 in ATCC 25296) formed a monophyletic clade together with SigH proteins in *Mycobacterium tuberculosis* and corynebacteria and SigR in *Streptomyces coelicolor* (Fig. 1). Evidence in the literature indicates that SigH (or SigR) proteins play an important role as central regulators for disulphide control in actinobacteria and that the transcription of their genes is modulated by a regulatory system consisting of SigH (SigR) and the SigH (SigR)-specific, zinc-containing, disulphide-sensitive anti-sigma factor.[29,30] Consistent with the previous studies on *M. tuberculosis*,[30] the putative anti-sigma factor genes (RMDY18_07080 in DY-18 and ROTMU0001_0902 in ATCC 25296) seemed to form an operon with the ECF sigma factors (RMDY18_07090 and ROTMU0001_0903), respectively. These putative anti-sigma factor genes have a canonical HXXXCXXC motif (Fig. 2) known to respond to disulphide stress.[29,30] The conservation of this motif in anti-sigma factor suggests that the operon found in DY-18 and ATCC 25296 might be involved in disulphide regulation. Thus, the ECF sigma factor and the putative anti-sigma factor found in two strains of *R. mucilaginosa* have been designated SigH and anti-SigH (RshA), respectively.

Another ECF sigma factor found in ATCC 25296 (ROTMU0001_0778) did not belong to any ECF sigma factor branches discussed in this study (Fig. 1). A BLASTP search using this ECF sigma factor sequence as a query identified a highly homologous group of sigma factors (E-value $<10^{-100}$) within oral actinobacteria: *R. dentocariosa* (YP_003983674 and

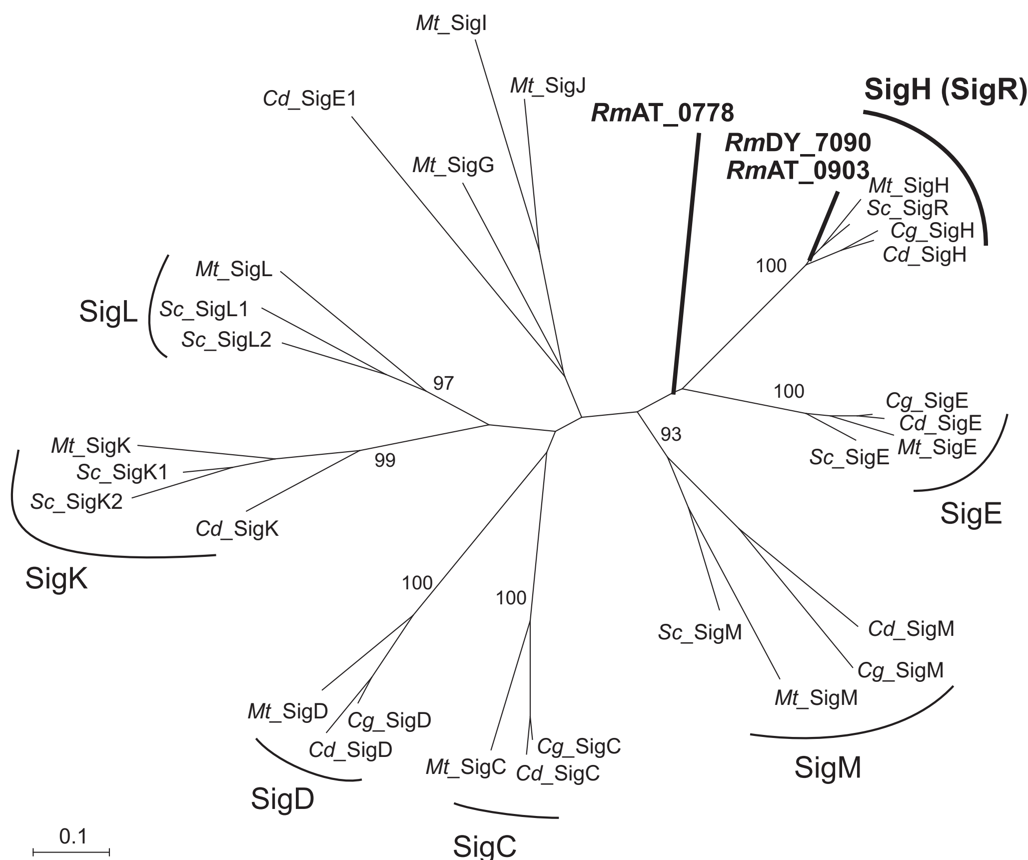

Fig. 1 – Phylogenetic tree of actinobacterial ECF sigma factors, including that of *R. mucilaginosa* DY-18 and ATCC 25296. The unrooted phylogenetic tree was inferred from aligned sigma factor sequences of *R. mucilaginosa* DY-18 (*Rm*DY), *R. mucilaginosa* ATCC 25296 (*Rm*AT), *Mycobacterium tuberculosis* H37Rv (*Mt*), *Streptomyces coelicolor* A3(2) (*Sc*), *Corynebacterium diphtheriae* NCTC 13129 (*Cd*), and *Corynebacterium glutamicum* ATCC 13032 (*Cg*). For several nodes of interest, bootstrap values are indicated at the nodes. The GenBank accession numbers for ECF sigma factors used are as follows: *Rm*DY_7090, BAI64541; *Rm*AT_0778, ZP_05368009; *Rm*AT_0903, ZP_05368129; Mt_SigC, CAA98224; Mt_SigD, CAB01009; Mt_SigE, CAB10918; Mt_SigG, CAB09733; Mt_SigH, CAB08314; Mt_SigI, CAA15866; Mt_SigJ, CAA17100; Mt_SigK, CAA17402; Mt_SigL, CAA17502; Mt_SigM, CAA16224; *Cd*_SigC, NP_938668; *Cd*_SigD, NP_938953; *Cd*_SigE, NP_939356; *Cd*_SigE1, NP_939244; *Cd*_SigH, NP_939083; *Cd*_SigK, NP_938496; *Cg*_SigC, NP_599507; *Cg*_SigD, NP_599836; *Cg*_SigE, NP_600348; *Cg*_SigH, NP_599995; *Cg*_SigM, NP_602281; Sc_SigE, NP_629295; Sc_SigR, NP_629363; Sc_SigK1, NP_625996; Sc_SigK2, NP_624943; Sc_SigL1, NP_627178; Sc_SigL2, NP_625240; Sc_SigM, NP_628078. Scale bar, 0.1% amino acid substitution.

ZP_07072575), *Actinomyces oris* (ZP_08126557), *A. viscosus* (ZP_08233082), *A. odontolyticus* (ZP_02045262, ZP_06607845) and *Actinomyces* sp. oral taxon (ZP_08033568, ZP_08293716).

3.2. *Diamide stress-mediated induction of sigH*

Next, we addressed the issue of whether the operon found in *R. mucilaginosa* is actually involved in disulphide regulation. We compared the mRNA levels of several genes including *sigH* before and after exposure to diamide by qRT-PCR in *R. mucilaginosa* DY-18 and ATCC 25296. The relative quantity of the target mRNA was normalised to the level of *gyrB* (RMDY18_00040) encoding the subunit of gyrase as an internal standard and the expression levels of target genes before and after treatments were then compared. The mRNA level of *sigH* in DY-18 cultures with diamide treatment showed more than 100-fold increase compared to those of untreated cultures. The transcriptional levels of *sigA* in DY-18 cultures with diamide were 10-fold higher than those of cultures without diamide (Fig. 3A). Similar results were obtained in the experiments with ATCC 25296 (Fig. 3B). The mRNA levels of *rpoB* as controls did not show significant changes in both strains (Fig. 3). The mRNA level of another ECF sigma factor in ATCC 25296 (ROTMU0001_0778) did not show significant changes after the diamide treatment (Fig. 3B).

3.3. *Putative SigH binding site and transcription start sites in the upstream region of sigH*

Primer extension assay revealed that *R. mucilaginosa* DY-18 had two transcription start sites in the upstream region of *sigH* and one site in the upstream region of *sigA* when the cells were exposed to the diamide stress (Fig. 4A). When the cells were not exposed to diamide, we could not detect any signals

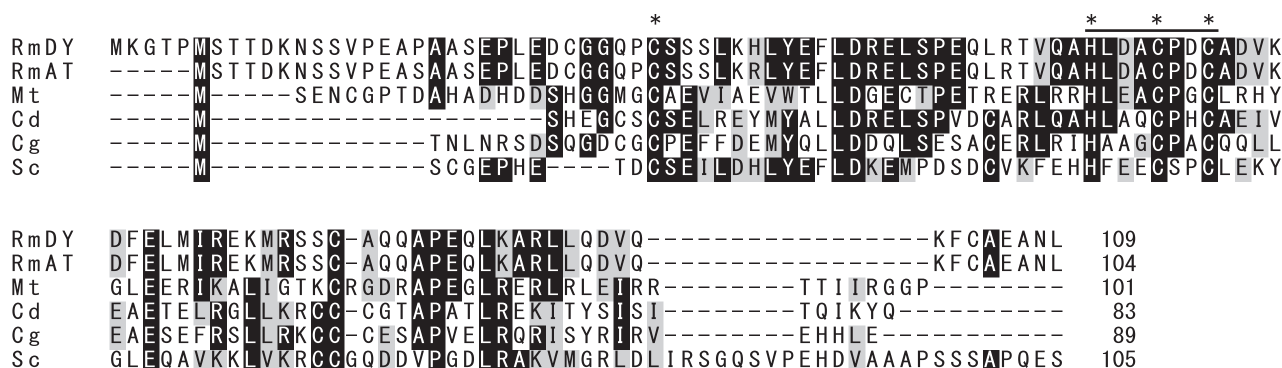

Fig. 2 – Protein sequence alignment of anti-SigH (SigR). Conserved cysteines and the canonical HXXXCXXC motif are indicated by asterisks and a line, respectively. Conserved residues are shaded in gray, and highly conserved residues are displayed in white against black background. MAFFT was used to create the alignment. The length of each protein is listed at the end of the alignment. Accession numbers for anti-sigma factors are as follows: RmDY (RMDY18_07080 of *R. mucilaginosa* DY-18), YP_003362360; RmAT (ROTMU0001_0902 of *R. mucilaginosa* ATCC 25296), ZP_05368128; Mt (Rv3221A of *M. tuberculosis*), YP_177945; Cd (DIP0710 of *C. diphtheriae*), NP_939084; Cg (cg0877 of *C. glutamicum*), YP_225058; and Sc (SCO5217 of *S. coelicolor*), NP_629364.

indicating the transcription start sites in the upstream regions of *sigH* and *sigA* in both strains (data not shown). The consensus sequences for the putative SigH binding site, which is known to be conserved in *M. tuberculosis* and *S. coelicolor*,[31,32] were found at −10 and −35 nt from the *sigH* transcription start site (Fig. 4B). In contrast, the putative SigH binding site did not exist in an appropriate position in the upstream region of *sigA* (Fig. 4B).

3.4. Microarray analysis of gene expression patterns under the diamide stress

To verify that the operon consisting of *sigH* and *rshA* is involved in the response against diamide stress and to see what kind of genes are up- or down-regulated in this event, we conducted a microarray analysis using a whole genome chip for *R. mucilaginosa* DY-18. The transcription levels of *sigH* and *rshA* were up-regulated at least 4.9-fold (in base 2 logarithm) in the cultures with diamide stress as compared to those of cultures without diamide (Table 2). The transcription level of *sigA* in the diamide-treated cultures showed a 3.5-fold increase. The data also demonstrated that several oxidative- and heat-stress-related genes were significantly up-regulated under the diamide stress. For example, genes encoding thioredoxins (RMDY_19830 and RMDY_08700) and mycothiol metabolic enzyme (RMDY_13730) (Table 2), which are known as major antioxidant systems under the control of SigH in actinobacterial species,[33,34] and the gene encoding OsmC (RMDY18_05850), which play a role in the protection of mycobacteria against oxidative stress,[35] are significantly

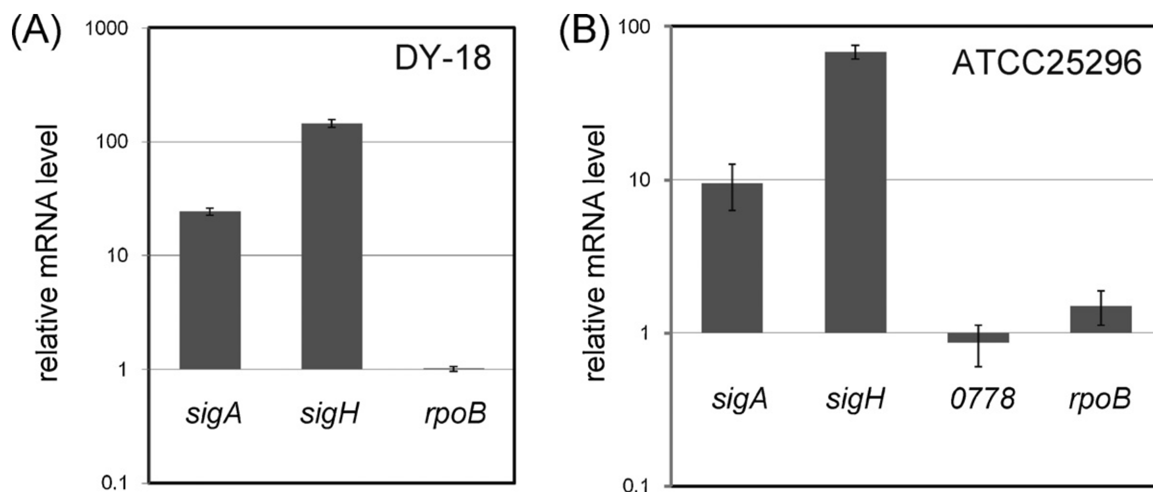

Fig. 3 – Relative mRNA levels of *sigH*, *sigA*, and *rpoB* after exposure to oxidative stress caused by diamide in DY-18 and ATCC25296. After the application of 5 mM diamide for 30 min, the cells were washed with TSB-Y medium and total RNA was extracted. The relative amounts of transcripts in DY18 (A) and ATCC25296 (B) were subsequently determined by real-time RT-PCR and calculated in relation to that of an untreated control culture. Experiments were carried out with three biological replicates. 0778: ROTMU0001_0778.

Fig. 4 – Putative SigH binding and transcription starting sites in upstream of *sigH*. (A) Primer extension electrophoregrams of the upstream regions of *sigH* and *sigA*. (B) The sequences of the *sigH* and *sigA* upstream regions are shown, and transcription initiation sites are indicated with bent arrows. Lines under the sequences correspond to the putative SigH-dependent promoters. Double lines under the sequences correspond to the start codon of *sigH* or *sigA* genes. The consensus sequences recognized by SigH of *M. tuberculosis* and SigR of *S. coelicolor* are shown at the bottom. Nucleotides of the consensus sequence conserved in more than 80% of the sequences are in capital letters.

up-regulated. In addition, seven genes, encoding reductase or oxidoreductase involved in transmitting electrons from one molecule to another, and three genes, concerned with transport system or transcriptional regulation, showed up-regulated transcriptional levels (Table 2). In contrast, the diamide treatment down-regulated several genes encoding the H^+-translocating adenosine triphosphatase (ATPase) (FoF1 ATPase) (Table 2).

4. Discussion

Sigma factors are crucial for prokaryotic transcription initiation where different sigma factors are activated in response to various environmental changes and which enable RNA polymerase binding to promoters of genes suitable for the circumstance.[21,36] The number of sigma factors varies among bacteria; for example, obligate symbionts *Chlamydia* and *Rickettsia* do not have ECF sigma factors. *Borrelia*, *Mycoplasma* and *Aquifex*, whose genome size is relatively small (less than 2 Mb), exist without ECF sigma factors also.[37] In contrast, 40–50 ECF sigma factors per genome are encoded in Acidobacteria, Actinobacteria and Bacteroidetes, and 103 ECF sigma factors in the *Catenulispora acidiphila* genome (Actinobacteria) should be described (MiST2 database) as a particular case. *M. tuberculosis*, which belongs to the Actinobacteria, has 10 sigma factors of the ECF sub-family among 13 sigma factors in total. These factors enable this bacterium to cope with various environmental conditions.[21,38] Some ECF sigma factors, such as SigD, SigE, SigC and SigH, are involved in the virulence of *M. tuberculosis*.[38–40] In this study, we attempted to characterise the sigma factors of *R. mucilaginosa*, which belongs to Actinobacteria and has an extremely low number of ECF sigma factors (one in DY-18 and two in ATCC 25296) as compared to most of the other strains. It is plausible that *R. mucilaginosa* might modulate their virulence and/or gene expressions for survival

Table 2 – Genes induced or repressed by diamide stress.

Category and gene	Ratio	Product
Induced genes		
RMDY18_05850	8.4	OsmC-like protein
RMDY18_00990	7.7	Conserved hypothetical protein
RMDY18_16680	7.5	Hypothetical protein
RMDY18_12060	6.6	Peptide methionine sulfoxide reductase
RMDY18_00970	6.6	Hypothetical protein
RMDY18_18240	6.4	Hypothetical protein
RMDY18_14560	6.4	Helix-turn-helix containing protein
RMDY18_02510	6.4	Pyridine nucleotide-disulphide oxidoreductase
RMDY18_00980	6.3	Heavy-metal-associated protein
RMDY18_18550	6.2	L-Cystine transport system permease protein, TcyB
RMDY18_04020	6.2	Flavin dependant oxidoreductase
RMDY18_07080	**6.2**	**Anti-sigma factor, RshA**
RMDY18_14550	6.1	Hypothetical protein
RMDY18_16690	6.1	Oxidoreductase, aldo/keto reductase family protein
RMDY18_18530	6.1	Hypothetical protein
RMDY18_19830	6.0	Thioredoxin
RMDY18_19810	5.9	Hypothetical protein
RMDY18_02650	5.8	Iron dependent repressor
RMDY18_19820	5.8	Pyridine nucleotide-disulphide oxidoreductase
RMDY18_00960	5.7	Hypothetical protein
RMDY18_00670	5.6	Acyl-CoA dehydrogenase
RMDY18_00680	5.6	Hypothetical protein
RMDY18_09110	5.4	DsbA oxidoreductase, FrnE
RMDY18_00690	5.4	Conserved hypothetical protein
RMDY18_18100	5.3	RNase H
RMDY18_08700	5.3	DsbA-like thioredoxin
RMDY18_18560	5.1	L-Cystine transport system, binding protein
RMDY18_07320	5.1	Conserved hypothetical protein
RMDY18_00830	5.0	Arsenical resistance operon repressor, ArsD
RMDY18_11060	5.0	Conserved hypothetical protein
RMDY18_08690	4.9	Hypothetical protein
RMDY18_15670	4.9	SUF system FeS assembly protein, NifU family
RMDY18_07090	**4.9**	**RNA polymerase sigma factor, SigH**
RMDY18_10160	4.9	Chaperone protein, DnaJ
RMDY18_12310	4.8	Methionine sulfoxide reductase
RMDY18_11630	4.7	Hypothetical protein
RMDY18_18540	4.6	Amino acid ABC transporter, ATP-binding protein
RMDY18_11640	4.6	6,7-Dimethyl-8-ribityllumazine synthase
RMDY18_18400	4.6	NAD dependent sugar epimerase/dehydratase family
RMDY18_13730	4.5	Mycothione reductase
Repressed genes		
RMDY18_12900	−6.3	ATP synthase subunit B (FoF_1)
RMDY18_12890	−6.1	Hypothetical protein
RMDY18_04530	−6.1	Nitrate reductase delta subunit
RMDY18_12870	−5.8	Hypothetical protein
RMDY18_04500	−5.5	Molybdopterin oxidoreductase
RMDY18_12910	−5.3	ATP synthase subunit C (FoF_1)
RMDY18_12880	−5.1	ATP synthase delta subunit (FoF_1)
RMDY18_12860	−5.0	ATP synthase alpha/beta family (FoF_1)
RMDY18_12850	−4.8	ATP synthase gamma subunit (FoF_1)

Relative ratios of the transcript levels after diamide treatment determined by DNA microarray analyses are shown in the base 2 logarithm. Genes encoding sigma and anti-sigma factors are indicated in bold.
Annotation based on BLASTP searches (http://www.ncbi.nlm.nih.gov/BLAST/) and Pfam 25.0 (http://pfam.sanger.ac.uk/).

by the limited number of ECF sigma factors. Based on the *in silico* analysis in this study, the ECF sigma factor in DY-18 (RMDY18_07090) and ATCC 25296 (ROTMU0001_0903) was designated as SigH, which is known as a central regulator of disulphide control in other actinobacteria such as *Mycobacterium* and *Streptomyces*.[41] Previous studies evidently showed that the transcriptional level of *sigH* is markedly up-regulated by diamide, the thiol-specific oxidating agent.[26,29] Judging from our data consistent with the previous study, the alternative sigma factor SigH of *R. mucilaginosa* could be a regulator in response to the disulphide state.

As the primary sigma factor, SigA, is believed to control the transcription of many housekeeping genes,[21,36] we hypothesised that the expression of *sigA* itself would not be affected significantly by environmental changes. However, our gene-expression studies with qRT-PCR and microarrays revealed that the expressions of *sigA* in two strains of *R. mucilaginosa* were up-regulated by diamide treatment as a lesser extent compared to the *sigH* expression. Primer extension assay did not find a putative binding site of SigH in the upstream region of *sigA*. Because of the limited number of the sigma factors in *R. mucilaginosa*, it is plausible that other regulatory mechanisms, such as a one- or two-component system, might be involved in redox response and then indirectly up-regulate the expression of housekeeping sigma factor SigA, although relays of signal transduction are unknown.

A DNA microarray of the *R. mucilaginosa* revealed 40 genes with more than 4.5-fold (in base 2 logarithm) enhanced expression after diamide treatment, including genes encoding functions in the thioredoxin family and OsmC, which are known as major antioxidant systems under the control of SigH in actinobacterial species.[41] Further, several genes encoding reductase, oxidoreductase, transporter or transcriptional regulator showed up-regulated transcriptional levels (Table 2). These systems and proteins in *R. mucilaginosa* might be important for responding against disulphide stress.

A total of 10 genes exhibited greater than 4.5-fold (in base 2 logarithm) decreased expression; five of them were ATP synthase genes. In the case of *S. coelicolor*, these genes were down-regulated by diamide independently of the redox sigma factor regulation.[34] Although the mechanism behind the down-regulation of genes involved in ATP synthase in this organism is unknown, it is conceivable that *R. mucilaginosa* can use a strategy similar to that of *S. coelicolor* for resisting diamide stress.

We found that *R. mucilaginosa* has a limited number of ECF sigma factors, which are up-regulated under disulphide stress.

Our microarray data suggested that this organism has similar disulphide stress responses to those of Mycobacteria whose system against oxidative stress is well studied.[33,42] In *Mycobacterium*, other stress response systems such as one-component and two-component systems and other multi-protein enzyme systems are known to be involved in their oxidative stress response.[42] Therefore, it is still to be elucidated how the relatively simple ECF sigma system in *R. mucilaginosa* shares roles with other stress response systems under oxidative stress. Two-component signal transduction and quorum sensing systems have been reported to be redox-controlled in *Streptococcus mutans*.[15–17] *Porphyromonas gingivalis*, a major periodontopathic bacterium, seems to have at least two systems against oxidative stress, OxyR-mediated and SigH-mediated systems.[18,19] Therefore, it is conceivable that substantial and complicated connection between different stress response systems might exist in *R. mucilaginosa*. We presume that elucidating such systems behind the phenomenon observed in this study might contribute to understand the reason why *R. mucilaginosa* is a member of various bacterial species that persist in root canal system and cause apical periodontitis.[43]

The function of another ECF sigma factor in ATCC 25296 (ROTMU0001_0778) could not be determined in this study. Because this ECF sigma factor and the factors from oral *Rothia* and *Actinomyces* species formed a highly homologous group, it is conceivable that these sigma factors have a common role to reside in the oral environment. Unfortunately, we also could not figure out the direct interaction between the gene expression events *via* SigH and the biofilm formation of this organism.[14] While a series of studies indicate that an ECF sigma factor AlgU (σ^E) and an anti-sigma factor MucA regulate biofilm formation of *Pseudomonas aeruginosa*, which is a prototype of biofilm-forming bacteria,[44–46] recent studies have shown that the regulation of biofilm formation in a variety of bacterial species occurs *via* bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), a ubiquitous bacterial second messenger.[47,48] Therefore, it is still controversial whether the sigma factor-dependent induction of gene transcription is existent behind the synthesis of adhesins and biofilm matrix components of this organism. These issues should be addressed in our future studies using a suitable molecular tool that is capable of introducing specific targeted mutagenesis on *sigH* as well as genes highlighted in this study.

Fundings

This work was supported in part by Osaka Dental University Joint Research Funds (B08-01) and Grants-in-Aid for Young Scientists (B) (23792118, to T.N.; 24791975, to K.Y.) and Grants-in-Aid for Scientific Research (C) (23592724, to H.F.) from the Ministry of Education, Culture, Sports, Science and Technology.

Conflict of interest

None declared.

Ethical approval

Not required.

Acknowledgements

We are grateful to Mr. Kazuki Ogiso (Department of Periodontology, Graduate School of Dentistry, Osaka Dental University) for his excellent assistance with electron microscopy. Part of this research was performed at the Institute of Dental Research, Osaka Dental University.

REFERENCES

1. Collins MD, Hutson RA, Båverud V, Falsen E. Characterization of a *Rothia*-like organism from a mouse: description of *Rothia nasimurium* sp. nov. and reclassification of *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 2000;**50**(3):1247–51.
2. Stackebrandt E, Rainey FA, Ward-Rainey NL. Proposal for a new hierarchic classification system, Actinobacteria classis nov. *International Journal of Systematic and Evolutionary Microbiology* 1997;**47**(2):479–91.
3. Zaura E, Keijser BJF, Huse SM, Crielaard W. Defining the healthy ‘‘core microbiome’’ of oral microbial communities. *BMC Microbiology* 2009;**9**(1):259.
4. Takeshita T, Suzuki N, Nakano Y, et al. Relationship between oral malodor and the global composition of indigenous bacterial populations in saliva. *Applied and Environment Microbiology* 2010;**76**(9):2806–14.
5. Michels F, Colaert J, Gheysen F, Scheerlinck T. Late prosthetic joint infection due to *Rothia mucilaginosa*. *Acta Orthopaedica Belgica* 2007;**73**(2):263–7.
6. Vaccher S, Cordiali R, Osimani P, Manso E, de Benedictis FM. Bacteremia caused by *Rothia mucilaginosa* in a patient with Shwachman-Diamond syndrome. *Infection* 2007;**35**(3): 209–10.
7. Lee AB, Harker-Murray P, Ferrieri P, Schleiss MR, Tolar J. Bacterial meningitis from *Rothia mucilaginosa* in patients with malignancy or undergoing hematopoietic stem cell transplantation. *Pediatric Blood & Cancer* 2008;**50**(3):673–6.
8. Hodzic E, Snyder S. A case of peritonitis due to *Rothia mucilaginosa*. *Peritoneal Dialysis International* 2010;**30**(3): 379–80.
9. Morgan EA, Henrich TJ, Jarell AD, et al. Infectious granulomatous dermatitis associated with *Rothia mucilaginosa* bacteremia: A case report. *American Journal of Dermatopathology* 2010;**32**(2):175–9.
10. Kaasch AJ, Saxler G, Seifert H. Septic arthritis due to *Rothia mucilaginosa*. *Infection* 2011;**39**(1):81–2.
11. Zamakhchari M, Wei G, Dewhirst F, et al. Identification of *Rothia* bacteria as gluten-degrading natural colonizers of the upper gastro-intestinal tract. *PLoS ONE* 2011;**6**(9):e24455.
12. Vianna ME, Horz H-P, Gomes BPFA, Conrads G. Microarrays complement culture methods for identification of bacteria in endodontic infections. *Oral Microbiology and Immunology* 2005;**20**(4):253–8.
13. Sakamoto M, Siqueira JF, Rôças IN, Benno Y. Bacterial reduction and persistence after endodontic treatment procedures. *Oral Microbiology and Immunology* 2007;**22**(1): 19–23.

14. Yamane K, Nambu T, Yamanaka T, et al. Complete genome sequence of *Rothia mucilaginosa* DY-18: a clinical isolate with dense meshwork-like structures from a persistent apical periodontitis lesion. *Sequencing* 2010;**2010**:457236.
15. Wen ZT, Burne RA. LuxS-mediated signaling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation. *Journal of Bacteriology* 2004;**186**(9):2682–91.
16. Senadheera MD, Lee AWC, Hung DCI, Spatafora GA, Goodman SD, Cvitkovitch DG. The *Streptococcus mutans vicX* gene product modulates *gtfB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. *Journal of Bacteriology* 2007;**189**(4):1451–8.
17. Biswas I, Drake L, Erkina D, Biswas S. Involvement of sensor kinases in the stress tolerance response of *Streptococcus mutans*. *Journal of Bacteriology* 2008;**190**(1):68–77.
18. Meuric V, Gracieux P, Tamanai-Shacoori Z, Perez-Chaparro J, Bonnaure-Mallet M. Expression patterns of genes induced by oxidative stress in *Porphyromonas gingivalis*. *Oral Microbiology and Immunology* 2008;**23**(4):308–14.
19. Yanamandra SS, Sarrafee SS, Anaya-Bergman C, Jones K, Lewis JP. Role of the *Porphyromonas gingivalis* extracytoplasmic function sigma factor, SigH. *Molecular Oral Microbiology* 2012;**27**(3):202–19.
20. Hahn M-Y, Raman S, Anaya M, Husson RN. The *Mycobacterium tuberculosis* extracytoplasmic-function sigma factor SigL regulates polyketide synthases and secreted or membrane proteins and is required for virulence. *Journal of Bacteriology* 2005;**187**(20):7062–71.
21. Sachdeva P, Misra R, Tyagi AK, Singh Y. The sigma factors of *Mycobacterium tuberculosis*: regulation of the regulators. *FEBS Journal* 2010;**277**(3):605–26.
22. Ulrich LE, Zhulin IB. The MiST2 database: a comprehensive genomics resource on microbial signal transduction. *Nucleic Acids Research* 2010;**38**(Database issue):D401–7.
23. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *Journal of Molecular Biology* 1990;**215**(3):403–10.
24. Katoh K, Toh H. Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics* 2010;**26**(15):1899–900.
25. Nambu T, Inagaki Y, Kutsukake K. Plasticity of the domain structure in FlgJ, a bacterial protein involved in flagellar rod formation. *Genes and Genetic Systems* 2006;**81**(6):381–9.
26. Paget MS, Kang JG, Roe JH, Buttner MJ. SigmaR, an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in *Streptomyces coelicolor* A3(2). *EMBO Journal* 1998;**17**(19): 5776–82.
27. Lloyd AL, Marshall BJ, Mee BJ. Identifying cloned *Helicobacter pylori* promoters by primer extension using a FAM-labelled primer and GeneScan analysis. *Journal of Microbiological Methods* 2005;**60**(3):291–8.
28. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 1987;**4**(4):406–25.
29. Kang JG, Paget MS, Seok YJ, et al. RsrA, an anti-sigma factor regulated by redox change. *EMBO Journal* 1999;**18**(15):4292–8.
30. Song T, Dove SL, Lee KH, Husson RN. RshA, an anti-sigma factor that regulates the activity of the mycobacterial stress response sigma factor SigH. *Molecular Microbiology* 2003;**50**(3):949–59.
31. Raman S, Song T, Puyang X, Bardarov S, Jacobs WR, Husson RN. The alternative sigma factor SigH regulates major components of oxidative and heat stress responses in *Mycobacterium tuberculosis*. *Journal of Bacteriology* 2001;**183**(20):6119–25.
32. Paget MS, Molle V, Cohen G, Aharonowitz Y, Buttner MJ. Defining the disulphide stress response in *Streptomyces coelicolor* A3(2): identification of the sigmaR regulon. *Molecular Microbiology* 2001;**42**(4):1007–20.
33. Mehra S, Kaushal D. Functional genomics reveals extended roles of the *Mycobacterium tuberculosis* stress response factor sigmaH. *Journal of Bacteriology* 2009;**191**(12):3965–80.
34. Kallifidas D, Thomas D, Doughty P, Paget MSB. The sigmaR regulon of *Streptomyces coelicolor* A32 reveals a key role in protein quality control during disulphide stress. *Microbiology* 2010;**156**(6):1661–72.
35. Saikolappan S, Das K, Sasindran SJ, Jagannath C, Dhandayuthapani S. OsmC proteins of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* protect against organic hydroperoxide stress. *Tuberculosis* 2011;**91**(Suppl 1):S119–27.
36. Pátek M, Nešvera J. Sigma factors and promoters in *Corynebacterium glutamicum*. *Journal of Biotechnology* 2011;**154**(2–3):101–13.
37. Staroń A, Sofia HJ, Dietrich S, Ulrich LE, Liesegang H, Mascher T. The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. *Molecular Microbiology* 2009;**74**(3): 557–81.
38. Manganelli R, Fattorini L, Tan D, et al. The extra cytoplasmic function sigma factor sigma(E) is essential for *Mycobacterium tuberculosis* virulence in mice. *Infection and Immunity* 2004;**72**(5):3038–41.
39. Sun R, Converse PJ, Ko C, Tyagi S, Morrison NE, Bishai WR. *Mycobacterium tuberculosis* ECF sigma factor *sigC* is required for lethality in mice and for the conditional expression of a defined gene set. *Molecular Microbiology* 2004;**52**(1):25–38.
40. Calamita H, Ko C, Tyagi S, Yoshimatsu T, Morrison NE, Bishai WR. The *Mycobacterium tuberculosis* SigD sigma factor controls the expression of ribosome-associated gene products in stationary phase and is required for full virulence. *Cellular Microbiology* 2005;**7**(2):233–44.
41. den Hengst CD, Buttner MJ. Redox control in actinobacteria. *Biochimica et Biophysica Acta* 2008;**1780**(11):1201–16.
42. Bhat SA, Singh N, Trivedi A, Kansal P, Gupta P, Kumar A. Mechanism of redox sensing in Mycobacterium tuberculosis. *Free Radical Biology & Medicine* 2012;**53**(8): 1625–41.
43. Siqueira Jr JF, Rôças IN. Clinical implications and microbiology of bacterial persistence after treatment procedures. *Journal of Endodontics* 2008;**34**(11). 1291-1301.e3.
44. Martin DW, Schurr MJ, Yu H, Deretic V. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to sigma E and stress response. *Journal of Bacteriology* 1994;**176**(21):6688–96.
45. Potvin E, Sanschagrin F, Levesque RC. Sigma factors in *Pseudomonas aeruginosa*. *FEMS Microbiology Review* 2008;**32**(1):38–55.
46. Schurr MJ, Deretic V. Microbial pathogenesis in cystic fibrosis: co-ordinate regulation of heat-shock response and conversion to mucoidy in *Pseudomonas aeruginosa*. *Molecular Microbiology* 1997;**24**(2):411–20.
47. Hengge R. Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology* 2009;**7**(4):263–73.
48. Schirmer T, Jenal U. Structural and mechanistic determinants of c-di-GMP signalling. *Nature Reviews Microbiology* 2009;**7**(10):724–35.