Analysis of *Kytococcus sedentarius* Strain Isolated from a Dehumidifier Operating in a University Lecture Theatre: Systems for Aerobic Respiration, Resisting Osmotic Stress, and Sensing Nitric Oxide

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Keywords

Actinobacteria · Halotolerance · Iron-sulfur cluster · Nitric oxide · Wbl-sigma factor interactions

Abstract

A strain of *Kytococcus sedentarius* was isolated from a dehumidifier operating in a university lecture theatre. Genome analysis and phenotypic characterisation showed that this strain, *K. sedentarius* MBB13, was a moderately halotolerant aerobe with a branched aerobic electron transport chain and genes that could contribute to erythromycin resistance. The major compatible solute was glycine betaine, with ectoine and proline being deployed at higher osmolarities. Actinobacteria possess multiple WhiB-like (Wbl) regulatory proteins, and *K. sedentarius* MBB13 has four (WhiB1, WhiB2, WhiB3, and WhiB7). Wbls are iron-sulfur proteins that regulate gene expression through interactions with RNA polymerase sigma factors and/or other regulatory proteins. Bacterial two-hybrid analyses suggested that WhiB1 and WhiB2, but not WhiB3 and WhiB7, interact with the C-terminal domain of the major sigma factor, σ^A ; no interaction was detected between any of the Wbl proteins and the only alternative sigma factors, σ^B , σ^H , or σ^J . The interaction between σ^A and WhiB1 or WhiB2 was disrupted in a heterologous system under growth conditions that produce nitric oxide and the

iron-sulfur clusters of the isolated WhiB1 and WhiB2 proteins reacted with nitric oxide. Thus, *K. sedentarius* strain exhibits the major phenotypic characteristics of the type strain and a comprehensive examination of the interactions between its four Wbl proteins and four sigma factors suggested that the Wbl proteins all operate through interaction with σ^A .

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Introduction

Several reports have suggested that human health is likely to be adversely affected by airborne bacteria circulating within the enclosed environment and that the source of these bacteria is mainly human occupants of these spaces (reviewed by Fujiyoshi et al. [2017]). There are two core methods of evaluating the levels of airborne bacteria in an indoor environment. Passive methods expose petri dishes containing solid culture medium to the air. Bacteria that settle from the atmosphere onto the surface of the plate are thus collected and subsequent incubation of the covered plates allows the airborne bacteria collected during the exposure period to be quantified. Alternatively, active methods physically expose liquid or a solid culture media to a known volume of air. Subsequent culturing allows measurement of the bacteria trapped in

this way [Napoli et al., 2012]. We were interested in the prevalence of antibiotic-resistant bacteria in the air of a university lecture theatre. To actively sample the air in the lecture theatre, a dehumidifier was prepared so that after operation the airborne bacteria that had collected in the water trap could be cultured. Seven bacterial isolates were identified by 16S rRNA gene sequencing and their antibiotic resistance profiles were determined. One of the seven, *Kytococcus sedentarius* MBB13, was resistant to three antibiotics (ciprofloxacin, erythromycin, and gentamicin) and hence this isolate was subjected to further investigation.

K. sedentarius was first isolated in 1944 from a slide immersed in sea water near San Diego, USA and has since been isolated from human skin and commercial airline cabin air [Osman et al., 2008; Sims et al., 2009]. *K. sedentarius* is a strictly aerobic, coccoid, gram-positive actinobacterium [Sims et al., 2009]. One characteristic of actinobacteria is the possession of Wbl (WhiB-like) iron-sulfur proteins [Bush, 2018]. The main structural features of Wbl proteins are the four invariant cysteine residues that coordinate a [4Fe-4S] cluster and a conserved five-residue G[V/I]WGG motif [Kudhair et al., 2017; Bush, 2018]. Most actinobacteria possess more than one Wbl protein. The seven Wbl proteins of *Mycobacterium tuberculosis* have been extensively studied. WhiB1 is an essential nitric oxide-responsive transcription regulator [Smith et al., 2010; Kudhair et al., 2017]. WhiB2 is also essential and likely has a role in coordinating cell division [Gomez and Bishai, 2000]. WhiB3 has an oxygen- and nitric oxide-sensitive [4Fe-4S] cluster and it is thought to be involved in redox homeostasis when *M. tuberculosis* is in a host phagosome and thus is implicated in virulence and persistence [Singh et al., 2007]. The iron-sulfur cluster of WhiB4 is sensitive to oxygen and nitric oxide, and WhiB4 likely regulates the response to oxidative stress because deletion of *whiB4* resulted in over-induction of anti-oxidant activities and enhanced resistance to oxidative stress [Chawla et al., 2012]. The *M. tuberculosis whiB5* mutant was attenuated in a mouse model of infection, was unable to resume growth following a chronic infection, and was more sensitive to nitric oxide than the parental strain [Casonato et al., 2012]. WhiB6, is implicated in pathogenesis through its regulation of the virulence critical Type VII secretion system ESX-1 and aspects of aerobic and anaerobic metabolism [Chen et al., 2016]. Lastly, WhiB7 is associated with resistance to several antibiotics that target translation [Lee et al., 2020]. Thus, Wbl proteins are implicated in a wide range of processes that are critical to pathogenesis and evidence suggests that most Wbl pro-

teins act as transcriptional regulators that exert their influence on gene expression at least in part through interaction with the housekeeping sigma factor [Burian et al., 2013; Feng et al., 2016; Kudhair et al., 2017; Wan et al., 2020].

Whilst generally considered to be part of the normal human commensal microflora, some strains of *K. sedentarius* are opportunistic pathogens being associated with pitted keratolysis [Longshaw et al., 2002], haemorrhagic pneumonia in acute myeloid leukaemia patients [Levenga et al., 2004], cerebral cyst, and ventriculoatrial shunt infections for those with congenital hydrocephalus [Greene et al., 1980]. It has been reported that all tested *K. sedentarius* strains were sensitive to streptomycin, novobiocin, tetracycline, neomycin, vancomycin, and polymyxin B and most were sensitive to erythromycin, kanamycin, and chloramphenicol [Stackebrandt et al., 1995]. The observation that *K. sedentarius* MBB13 was resistant to erythromycin and that topical application of erythromycin is widely used to treat clinical *K. sedentarius* pitted keratolysis [Pranteda et al., 2014] prompted further investigation of the properties of the *K. sedentarius* MBB13 isolate.

Materials and Methods

Bacterial Isolation and Genomic DNA Analysis

A dehumidifier (Meaco 12L Platinum) was operated for 1 h in a University of Sheffield lecture theatre at the end of the teaching week. Water collected in the dehumidifier was transferred to a 50 mL Falcon tube and centrifuged. The supernatant was discarded and the liquid that remained (~50 µL) was streaked on a Nutrient Agar (NA) plate and incubated at 37°C for 18 h. The following day, colonies were sub-cultured on fresh NA plates and were incubated at 37°C for 18 h. One colony of each sub-cultured bacterium was inoculated into 5 mL Nutrient Broth (NB) in 50 mL Falcon tubes and incubated at 37°C for 18 h.

For 16S RNA gene sequencing universal bacterial primers (F-27 and R-1492; online supplementary Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000512751) were used to produce amplified DNA fragments (~1.5 kb). The DNA sequences of the PCR products were analysed using the BLAST programme available through the National Centre for Biotechnology Information (NCBI) to identify the closest matches in the database. For *K. sedentarius* MBB13 the match to the reference strain DSM 20547 (541T) was 97% identical.

Genomic DNA extraction was carried out using either Sigma GenElute or Qiagen QIAamp DNA mini kits according to the manufacturer's instructions. An additional step, to improve cell lysis of *K. sedentarius* MBB13 was treatment of the cell suspensions with lysozyme (300 µg/mL), achromopeptidase (200 µg/mL), lyso-staphin (200 µg/mL), mutanolysin (100 µg/mL), and proteinase K (40 µL of a 20 mg/mL solution) for 30 min at 37°C [Sims et al., 2009].

Genomic DNA was sequenced by MicrobesNG (University of Birmingham, UK). Four trimmed files (fastq.gz) were used to generate three files for analysis in the ACT software [Carver et al., 2005], namely: (1) Galaxy-[Concatenated_reference_Genbank_file], (2) Galaxy-[ACT_comparison_file].tabular, and (3) Galaxy-[Concatenated_contigs_Genbank_file].

Bacterial Growth

Bacterial strains and plasmids and their sources are listed in (online supplementary Table 2). *K. sedentarius* MBB13 cultures were routinely grown aerobically in Luria-Bertani (LB) broth, with orbital shaking (250 rpm) at 37°C overnight. The ability to grow in the absence of oxygen was assessed by incubating LB streaked with *K. sedentarius* MBB13 in anaerobic jars at 37°C for 18 h. In addition, thioglycollate broth was used [Tille, 2014]. Single colonies of *K. sedentarius* MBB13 or *Escherichia coli* MG1655 were picked from LB plates and placed into sterilised thioglycollate broth (10 mL) in 25 mL glass universal tubes. The tubes were sealed and incubated at 37°C for 48 h.

For preparation of competent cells, overnight cultures (5 mL) of either *E. coli* or *Mycobacterium smegmatis* were grown in LB or 7H9 broth (Sigma-Aldrich), respectively. A 250 mL flask containing either LB or 7H9 (50 mL) was inoculated with 300 µL of the overnight culture of *E. coli* or *M. smegmatis*. Cultures were incubated at 37°C with shaking at 250 rpm until the optical density at 600 nm (OD₆₀₀) reached ~0.6 (*E. coli*, ~2 h) or ~0.9 (*M. smegmatis*, ~18 h). The cultures were immediately placed on ice for 30 min for *E. coli* or ~2 h for *M. smegmatis*. The bacteria were then collected by centrifugation at 3,500 g for 10 min at 4°C. The supernatant was discarded and the cell pellets were suspended in 10% (v/v) cold glycerol and centrifuged at 3,500 g for 10 min at 4°C. This process was repeated twice. The cell pellets were suspended in 1,000 µL 10% (v/v) cold glycerol then, aliquots (100 µL) were transferred to pre-cooled Eppendorf tubes in dry ice and stored at -80°C.

For initial antibiotic resistance profiling autoclaved ISO agar medium was prepared and left to cool to ~45°C before bacterial suspensions were added to match the turbidity of the 0.5 McFarland standard (0.111 OD₆₀₀). The agar was poured into petri dishes (30 mL per plate) and, after setting, antibiotic discs were placed at the centre of the plates. The plates were incubated at 37°C for 18 h. Measurement of antibiotic inhibition zones and interpretation of the data was carried out according to the British Society for Antimicrobial Chemotherapy guidelines (BSAC). Minimum inhibitory antibiotic concentrations for growth of *K. sedentarius* MBB13 was carried out in ISO broth medium in 96 well plates. Bacterial suspensions (100 µL at 0.5 McFarland standard; 0.111 OD₆₀₀) were added to ISO broth medium (100 µL) containing 2,048 µg mL⁻¹ of the indicated antibiotic. Then 100 µL of this suspension was used in serial dilutions until a final concentration of 1 µg mL⁻¹. Data interpretation was according to the BSAC guidelines.

Sample Preparation for Nuclear Magnetic Resonance (NMR)

Overnight cultures (50 mL in 250 mL conical flasks) of *K. sedentarius* MBB13 were grown in Nutrient broth containing the indicated concentrations of NaCl at 37°C for 16 h with shaking (250 rpm). Bacteria were collected by centrifugation for 10 min at 3,000 g. The supernatants were discarded and the pellets were resuspended in 1 mL dH₂O. The chilled bacterial suspensions were sonicated (3 × 20 s with 15 s cooling between each sonication step) to lyse the bacteria. Samples then were transferred to Eppendorf

tubes and centrifuged at 13,000 g for 7 min. The supernatants were transferred to pre-weighed Eppendorf tubes and frozen overnight at -80°C. The tubes were capped with lids that had been pierced with a needle and placed in a freeze-dryer for 72 h. After re-capping with the original unpierced lids, the tubes were re-weighed and stored at room temperature. For analysis, the samples were dissolved in 530 µL D₂O and 5 µL trimethylsilyl propionate (100 mM). NMR spectra were collected using a Bruker 600 MHz instrument.

Two-Hybrid Assay

The bacterial adenylate cyclase-based two-hybrid (BACTH) system was used to detect interaction between WhiB proteins (1, 2, 3, and 7) and sigma factors (A, B, H, and J) in vivo [Karimova et al., 1998]. The genes encoding the WhiB proteins and the sigma factors were amplified from via PCR using primers (online supplementary Table 1). PCR products were digested and ligated into pKT25 between the *Xba*I and *Kpn*I for *wbl* genes, and pUT18 between *Hind*III and *Kpn*I for the sigma factors. Thus, each Wbl protein was fused to the C-terminal of T25, and each sigma factor was fused to the N-terminal of T18 (online supplementary Table 3 and online supplementary Fig. 1). Due to amplification difficulties, *whiB3*, σ^A C-terminal domain (CTD), and σ^B were optimized to *E. coli* K-12 and synthesised, and σ^J was synthesised and ligated into pUT18 by Eurofins (gene optimization was done using this webpage: http://gcua.schoedl.de/sequential_v2.html). Plasmids were propagated in *E. coli* DH5α. The plasmids that encode the T25-WhiB and Sigma-T18 protein fusions were used to co-transform electrocompetent *E. coli* BTH101. Transformed cells were plated on LB agar supplemented with ampicillin (100 µg/mL), streptomycin (50 µg/mL), and kanamycin (35 µg/mL), and incubated at 37°C for 24–36 h. After electroporation, one colony was picked and transferred to LB broth (5 mL) containing appropriate antibiotics and incubated overnight at 37°C. Samples (10 µL) of the cultures were dropped onto MacConkey/maltose plates supplemented with maltose (1%), the appropriate antibiotics, and IPTG 0.5 mM (for induction) and incubated at 30°C for 36–40 h.

Purification of WhiB1 and WhiB2 Expressed in *M. smegmatis*

The coding regions of *whiB1* and *whiB2* were amplified from *K. sedentarius* MBB13 genomic DNA by PCR (online supplementary Table 1). The products were digested and ligated into pMyNT (Hygromycin-B “Hygro-BR”) between the *Nco*I and *Hind*III sites for heterologous overproduction of the corresponding proteins in *M. smegmatis* mc²155 (online supplementary Table 3). The integrity of both plasmids was checked by DNA sequencing. Plasmids containing *whiB1* (JRG6949) and *whiB2* (JRG6947) were purified and used to transform *M. smegmatis* mc²155 (JRG6161). Protein overproduction was carried out in 2 L flasks containing 7H9 medium with Hygro-BR (100 µg/mL). Cultures were grown at 37°C to OD₆₀₀ ~0.9 (~18 h) and then induced with acetamide (0.2%) and incubated at 37°C for ~12 h, with shaking (250 rpm). Cells were collected by centrifugation at 17,500 g for 30 min and stored at -20°C. Frozen cells were suspended in either 20 mM NaH₂PO₄, pH 7.4, or 50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl, and disrupted by sonication. Cells were sonicated (9 × 25 s pulses) on ice by Soniprep 150 Plus set to the maximum amplitude. The sonicated samples were clarified by centrifugation at 45,500 g for 20 min. The His-tagged WhiB1 and WhiB2 proteins were isolated from the extracts (10 mL) by application to 5 mL HiTrap chelating

Fig. 1. Genome comparison overview of *K. sedentarius* MBB13 and DSM 20547. **a** Linear diagram generated by the Galaxy assembly website (<https://hactar.shef.ac.uk/galaxy>). Arrows at the bottom indicate the position of contigs, red lines indicate conserved regions with the same polarity in both genomes, and blue lines indicate conserved regions with opposite polarity. **b** Circular diagram. The burgundy circle indicates the reference genome, and the green circle indicates the MBB13 genome. The %GC content is shown in an adjacent feature track (black circle). The GC skew is shown in the last circle from inside (green and violet). This CGView map is generated via the online website of http://stothard.afns.ualberta.ca/cgview_server/ [Grant et al., 2012].

columns using the manufacturer's standard procedure with a linear gradient (0–100% B) formed from binding buffer A (50 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl) and elution buffer B (buffer A containing 0.5 M imidazole). Eluted fractions were analysed by SDS-polyacrylamide gel electrophoresis to determine purity and those containing Wbl proteins were stored at 4°C.

Protein Concentration Measurement

Routinely, protein concentrations were measured by the Bradford assay (Bio-Rad) used according to the manufacturer's procedure. For pure proteins, the theoretical extinction coefficient of WhiB1 or WhiB2 was first obtained from the ExPASy webpage (<https://web.expasy.org/protparam/>), then absorbance at 280 nm was used to calculate the protein concentration.

Iron-Sulfur Cluster Reconstitution

All reconstitution steps were carried out inside the anaerobic cabinet (Don Whitley A85) at room temperature (~20°C) and 0% oxygen concentration. Buffers and other solutions were left for at least 18 h inside the cabinet to ensure that they were anaerobic. Iron-sulfur cluster reconstitution was achieved using the NifS-based system [Kudhair et al., 2017]. The reaction was left inside the anaerobic cabinet overnight. On the next day, the reconstituted WhiB2 was dialysed against anaerobic buffer: 25 mM NaH₂PO₄, 0.25 M NaCl, pH 7.4, overnight to remove non-integrated components. The dialysed, reconstituted WhiB2 was transferred into Hellma® quartz cuvette (10 mm pathlength) and the screw cap lid was sealed to maintain anaerobic conditions. The [4Fe-4S] cluster content was estimated by UV-visible spectroscopy using an extinction coefficient of 16,750 M⁻¹ cm⁻¹ at 420 nm [Kudhair et al., 2017].

Reaction of WhiB1 and WhiB2 with NO

Spermine NONOate (Cayman) was used to investigate the sensitivity of the WhiB1 and WhiB2 iron-sulfur clusters to NO. Spermine NONOate was prepared by dissolving the powder in water to the final concentration of 19.06 mM. Spermine NONOate solution (30 µL) was injected using a Hamilton syringe into 1 mL of anaerobic protein (~35 µM) in a sealed Hellma® quartz cuvette. The reaction was followed by obtaining UV-visible spectra (300–800 nm).

Results

The Genome Sequence of *K. sedentarius* MBB13

The genome of *K. sedentarius* MBB13 was sequenced and assembled using the *K. sedentarius* DSM 20547 (541T) genome as a reference. The DNA was sequenced with 30x coverage and 152 contigs were used to assemble a 2,695,411 bp genome, which had a GC content of 71.57% (Fig. 1). The assembled genome was annotated using PROKKA [Seemann, 2014] and was predicted to contain 2,499 genes of which 1,789 were assigned a predicted function with the remaining 710 genes encoding hypothetical proteins (Table 1).

Although the *K. sedentarius* MBB13 genome should be considered to be a draft assembly, 85 loci present in the

reference genome (*K. sedentarius* DSM 20547 (541T)) were unmatched in the MBB13 genome (online supplementary Table 6). Of these, 24 loci included genes coding for transposase/integrase family proteins, suggesting some element of horizontal gene acquisition. The unmatched regions contained 35 pseudogenes and 438 genes, of which 174 encoded either hypothetical or uncharacterised proteins. There were 27 unmatched transcriptional regulators and 4 unmatched σ^{70} -family sigma factors, suggesting that the unmatched loci incorporated at least some of the apparatus needed to integrate these genes into the core regulatory networks of *K. sedentarius* DSM 20547 (541T). *K. sedentarius* DSM 20547 (541T) has two *rrn* operons coding for the 5, 16, and 23S rRNA molecules and one of these was apparently absent from the *K. sedentarius* MBB13 genome assembly. A notable feature of the unmatched genes was the presence of genes encoding multi-drug-resistance pumps (10 genes in 9 unmatched loci) and systems associated with resistance to Cd(II), Hg(II), and Cu(II).

The draft *K. sedentarius* MBB13 genome contained 79 loci made up of a total of 359 genes that were not matched in the reference genome (online supplementary Table 7). Of these, 181 genes coded for hypothetical proteins. Twelve of 79 unmatched loci contained predicted transposase/integrase family proteins (7 of each), suggesting that these regions were acquired horizontally. The unmatched regions included 18 genes coding for transcription regulators (with a further 3 helix-turn-helix domain proteins) and 2 sigma factors, including the extracytoplasmic function (ECF) sigma factor σ^J . There were no unambiguous candidates (such as *erm* genes coding for 23S rRNA methylation proteins) [Weisblum, 1995] within the unmatched regions to account for the observed antibiotic resistance profile of *K. sedentarius* MBB13. However, amongst the predicted transcription regulators was a two-component system, LiaS/VraR, which in other gram-positive bacteria coordinates the response to cell wall-targeting antibiotics [Kuroda et al., 2003; Shankar et al., 2015]. This two-component system sits upstream of genes coding for a predicted daunorubicin/doxorubicin resistance ATP-binding protein DrrA, two ABC-2 family transporter proteins, a hypothetical protein, and a VanZ-like protein (online supplementary Table 7). Drr-type ABC-transporters contribute to moderate level macrolide resistance in *M. tuberculosis* complex bacteria [Choudhuri et al., 2002], and so this locus could be involved in the observed erythromycin resistance. Furthermore, VanZ membrane proteins are associated with resistance to lipoglycopeptide antibiotics [Vimberg et al.,

Table 1. Overall genome sequence composition of *K. sedentarius* MBB13 and DSM 20547

	MBB13	DSM 20547
Number of bases	2,695,411	2,785,024
GC percentage	71.55	71.63
Total genes	2,499	2,554
Assigned genes	1,789	1,963
Genes encoding hypothetical proteins	710	591

2020]. Interestingly, there is a second Drr-type system in *K. sedentarius* MBB13 that is apparently absent in DSM 20547 (541T). Other potential contributors to the observed antibiotic resistance profile of *K. sedentarius* MBB13 included a predicted streptothricin hydrolase and the multidrug efflux pumps Stp and QacA. The former could expand the range of antibiotic resistance by inactivating streptothricin and the latter by efflux of antibiotics. To determine the contributions, if any, of these unmatched genes to the observed resistance of *K. sedentarius* MBB13 to ciprofloxacin, erythromycin, and/or gentamicin will require further experiments with mutant strains.

K. sedentarius MBB13 Is an Aerobe

The *K. sedentarius* type strain is an obligate aerobe [Sims et al., 2009]. Whereas the facultative anaerobe *E. coli* exhibited growth throughout a thioglycollate broth medium, *K. sedentarius* MBB13 grew only at the surface in contact with air, indicating that *K. sedentarius* MBB13 is an aerobe (Fig. 2a). This conclusion was supported by assessing *K. sedentarius* MBB13 growth on solid medium under anaerobic and aerobic conditions (Fig. 2b). Analysis of the genome sequence indicated the presence of a complete glycolytic pathway and citric acid cycle, but the absence of genes associated with anaerobic fermentative or respiratory metabolism. Some strains of *K. sedentarius* are able to reduce nitrate to nitrite [Sims et al., 2009], a reaction which could allow nitrate to serve as an alternative terminal electron acceptor; however, the MBB13 strain did not possess genes resembling known nitrate reductases. In contrast, *K. sedentarius* MBB13 appeared to have genes coding for a branched aerobic respiratory chain beginning with NADH, succinate, and proline dehydrogenases and ending with cytochrome *bc1-aa3* and cytochrome *bd* oxidases, linked by the quinone MK8-12, to permit O₂ to be used as the terminal electron acceptor (Fig. 2c).

Fig. 2. *K. sedentarius* MBB13 is an aerobe. **a** Growth of *K. sedentarius* MBB13 was only observed at the interface between thioglycollate broth and the atmosphere (left), whereas growth of the facultative anaerobe, *E. coli* MG1655, was observed within the broth (right). **b** Growth of *K. sedentarius* MBB13 on LB agar under anaerobic (left) and aerobic (right) conditions. **c** *K. sedentarius* MBB13 has genes coding for a branched aerobic electron transport chain. The primary dehydrogenases, NADH (Ndh), succinate (Sdh), and proline (PutP/ProC) and their predicted H^+/e^- ratios are shown, along with the quinone (MK8-12) and the terminal oxidases cytochrome *bc1-aa3* and cytochrome *bd* and their predicted H^+/e^- ratios.

Glycine Betaine Is the Major Compatible Solute of *K. sedentarius* MBB13

The *K. sedentarius* type strain 541T can grow in media containing up to 10% (~1.7 M) NaCl [Sims et al., 2009]. The OD₆₀₀ measurements of *K. sedentarius* MBB13 cultured for 24 h in Nutrient broth supplemented with additional NaCl (nutrient broth contains 0.1 M NaCl) showed that growth negatively correlated with increased salinity (Fig. 3a). At NaCl concentrations >1.1 M growth was weak, suggesting that *K. sedentarius* MBB13 is only moderately halotolerant [Ara et al., 2013]. Many bacteria use compatible solutes to counteract the effects of high osmolarity environments [Wood et al., 2001]. Proton NMR analysis of cell extracts from *K. sedentarius* MBB13 cultures grown for 24 h in the presence of different NaCl concentrations showed that glycine betaine accumulated as the NaCl concentration increased (Fig. 3b). Proline appeared in the NMR spectra at NaCl concentrations >1.1 M. A species with chemical shift of ~4.1 ppm suggested that ectoine was produced when the NaCl concentration was >2.1 M.

Choline is a precursor of glycine betaine. Choline and glycine betaine uptake is mediated by the ABC-type

transporter OpuC, which is likely encoded by the *opuBA-opuCD-opuCB-opuCC-lcdH* operon in the *K. sedentarius* MBB13 genome; the *opuD* and *betL* genes, coding for Betaine/Carnitine/Choline Transporter (BCCT) family transporters, could also permit uptake of these solutes. In gram-positive bacteria conversion of choline to glycine betaine is a two-step process catalysed by choline dehydrogenase, GbsB and an aldehyde dehydrogenase, BetB [Wood et al., 2001; Wood, 2006]. Although a *betB* gene was identified in the *K. sedentarius* MBB13 genome, a *gbsB* ortholog was not detected. Ectoine is a major compatible solute in many halophiles, *K. sedentarius* MBB13 possesses an *ask-ect-asd-ectABCD* operon and thus has the necessary machinery for synthesis of the precursor, L-aspartate- β -semialdehyde (*ask-ect-asd*), and its subsequent conversion to ectoine/hydroxyectoine (*ectABCD*) (Fig. 3c). Proline and trehalose are widely used compatible solutes and *K. sedentarius* MBB13 possesses a proline transporter (*proP*) and trehalose synthesis enzymes (*otsAB*) [Kaasen et al., 1994] (Fig. 3c). Following osmotic down-shifts these accumulated compatible solutes need to be released to minimise water influx and *K. sedentarius* MBB13 has two mechanosensitive channels

Fig. 3. *K. sedentarius* MBB13 systems involved in osmotic adaptation. **a** Growth of *K. sedentarius* MBB13 in nutrient broth supplemented with different concentrations of NaCl at pH 7.5 ± 0.2 after 24 h incubation at 37°C on an orbital shaker (150 rpm). Optical density was measured at 600 nm (OD₆₀₀) against a medium blank. Data points are the means of three replicates plus or minus standard deviation. **b** Proton NMR spectra of cell extracts from *K. sedentarius* MBB13 grown in nutrient broth medium (containing 0.1 M NaCl) supplemented with 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 M NaCl. The spectra shown are equivalent to ~10 OD₆₀₀ units of bacteria without additional NaCl, ~8 OD₆₀₀ units of bacteria supplemented with 0.5, 1.0, or 1.5 M NaCl, ~6 OD₆₀₀ units of bac-

teria for cultures supplemented with 2.0 M NaCl, and 0.2 OD₆₀₀ units of bacteria for cultures supplemented with 3.0 M NaCl. Peaks associated with relevant compounds are indicated. **c** Analysis of the genome sequence identified transporters, channels, and biosynthetic pathways that could contribute to tolerating high salinity. Membrane proteins involved in release or uptake of environmental compatible solutes and cytoplasmic enzymes for the synthesis of compatible solutes (glycine betaine, ectoine, and trehalose) are shown. MscS and MscL are mechanosensitive channels which are transient gating systems to release compatible solutes in response to an osmotic down-shock, preventing cell lysis.

Fig. 4. Interaction of Wbl proteins and sigma factors (σ) using the BATCH system. **a** Patches of *E. coli* BTH101 co-transformed with pKT-25 and pUT18 coding for the indicated fusion proteins on MacConkey medium in the presence of maltose. The pKT25-zip and pUT18-zip plasmids served as a positive control (+) and the pKT25 and pUT18 vectors served as a negative control (-) (bottom row). **b** Interaction between fusion partners in liquid cultures. Data points are the mean values for β -galactosidase specific ac-

tivities from four cultures, plus or minus standard deviation; significance (*t* test) is indicated. **c** Nitric oxide impairs the WhiB1: σ^A and WhiB2: σ^A interactions. The data shown are β -galactosidase specific activities normalised to those obtained for the positive control (Zip-Zip) cultures grown under the indicated conditions. The chart shows the means and standard deviations for four independent cultures; significance (*t* test) is indicated.

(*mscL* and *ykuT/mscS*) to fulfil this function. Thus, analysis of the *K. sedentarius* MBB13 genome and the experiments presented here suggest that glycine betaine is the major compatible solute, but proline and ectoine also contribute to tolerance at higher NaCl concentrations.

K. sedentarius MBB13 Has Four Wbl Proteins That Interact with the Primary Sigma Factor, σ^A

One of the characteristics of actinobacteria is the possession of Wbl (WhiB-like) proteins [Bush, 2018]. Wbl proteins are small (~80–140 residues) with four invariant cysteine residues that coordinate a [4Fe-4S] cluster [Bush,

Fig. 5. The iron-sulfur clusters of *K. sedentarius* MBB13 WhiB1 and WhiB2 proteins react with nitric oxide. **a** UV-visible spectra of holo-WhiB1 (58 μ M cluster) before (solid line) and after (dashed line) exposure to Spermine NONOate (571.8 μ M). **b** UV-visible spectra of WhiB2 (25 μ M cluster) before (solid line) and after (dashed line) treatment with Spermine NONOate (571.8 μ M). The arrows indicate the direction of the absorbance changes. The treated traces were normalised to the untreated readings.

2018]. The iron-sulfur cluster acts as a redox sensor in controlling diverse biological processes, such as morphogenesis, metabolism, virulence, and antibiotic resistance, most likely by interacting with, and modulating the activity of, RNA polymerase sigma factors [Miller and Auerbuch, 2015; Kudhair et al., 2017; Wan et al., 2020]. *K. sedentarius* MBB13 possesses four Wbl encoding genes (*whiB1*, *whiB2*, *whiB3*, and *whiB7*) and four RNA polymerase sigma factors (*sigA*, *sigB*, *sigH*, and *sigI*) (online supplementary Fig. 2). All possible interactions between the Wbl proteins and sigma factors were tested using a bacterial two-hybrid (BACTH) system [Karimova et al., 1998]. Plating on indicator medium suggested that the WhiB1, B2, and B3 proteins interacted with the C-terminal domain of σ^A , but not with any of the other sigma factors; WhiB7 did not interact with any of the sigma factors (Fig. 4a). This conclusion was confirmed by measuring β -galactosidase activities of aerobic liquid cultures, although the WhiB3: σ^A interaction, whilst greater than the negative control ($p = 0.0741$), was weaker than in other protein combinations (Fig. 4b). Although these data suggest that the *K. sedentarius* MBB13 Wbl proteins only interact with σ^A , the possibility that the σ^B -T18, σ^H -T18, and/or σ^I -T18 fusion proteins were incorrectly folded and/or unstable, leading to false negative results, cannot be excluded.

Previous work had shown that the interaction between *M. tuberculosis* WhiB1 and σ^A was impaired in the pres-

ence of nitric oxide [Kudhair et al., 2017]. This was also the case for the *K. sedentarius* MBB13 WhiB1: σ^A and WhiB2: σ^A interactions (Fig. 4c). Recombinant expression and purification of the *K. sedentarius* MBB13 WhiB1 and WhiB2 proteins showed that the WhiB1 iron-sulfur cluster was stable under aerobic conditions, as judged by the colour of the isolated protein (straw brown) and its UV-visible spectrum being typical of a [4Fe-4S] protein (Fig. 5a). In contrast, the isolated WhiB2 protein was colourless and after anaerobic reconstitution the UV-visible spectrum indicated the presence of both [4Fe-4S] and [2Fe-2S] clusters, suggesting that the WhiB2 iron-sulfur cluster was less stable than that of WhiB1 (Fig. 5b). Consistent with the impaired interaction observed using the BACTH system, both WhiB1 and WhiB2 iron-sulfur clusters reacted with nitric oxide with changes in the UV-visible spectra (increased absorbance at 350–360 nm and decreased absorbance at 420 nm) suggesting the formation of a dinuclear iron dinitrosyl species known as Rousin's red ester [Kudhair et al., 2017; Crack and Le Brun, 2019] (Fig. 5).

Discussion

A strain of *K. sedentarius* (designated as MBB13) isolated from a dehumidifier operating in a university lecture theatre was found to be resistant to three antibiotics (cip-

rofloxacillin, erythromycin, and gentamicin; online supplementary Tables 4 and 5). Inspection of the *K. sedentarius* MBB13 draft genome suggested several possible contributors to this antibiotic resistance profile, including several efflux pumps, without revealing the source of the resistance to erythromycin, which was of particular interest because this antibiotic is often used to treat *K. sedentarius* infections. The isolated strain was an aerobe, with genes suggesting a central metabolic map consisting of glycolysis, citric acid cycle, and a branched aerobic electron transport chain. Some *K. sedentarius* strains have been reported to reduce nitrate, raising the possibility of anaerobic nitrate respiratory growth, but nitrate reductase genes were absent from the *K. sedentarius* MBB13 genome. *K. sedentarius* is found on the skin, mucosae, and oropharynx of human beings [Sims et al., 2009] and this is likely to be the source of the strain trapped by the dehumidifier. *K. sedentarius* MBB13 possessed several mechanisms for counteracting osmotic stress, including compatible solute uptake systems and enzymes for synthesis of ectoine and trehalose (Fig. 3c). It appeared that glycine betaine was the major compatible solute, but was supplemented at higher salt concentrations by ectoine and proline.

One of the characteristics of actinobacteria is the possession of Wbl proteins [Feng et al., 2016]. *K. sedentarius* MBB13 has four *wbl* genes similar to the *whiB1*, *whiB2*, *whiB3*, and *whiB7* of *M. tuberculosis* (online supplementary Fig. 2). The major *M. tuberculosis* sigma factor (σ^A) interacts with all the *M. tuberculosis* Wbl proteins except *WhiB5* [Feng et al., 2016]. *K. sedentarius* MBB13 has four sigma factors (σ^A , σ^B , σ^H , and σ^J) (online supplementary Fig. 2). Therefore, BACTH system was used to test all possible interactions between the *K. sedentarius* MBB13 Wbl proteins and the sigma factors. Significant interactions were identified for *WhiB1* and *WhiB2* with σ^A (Fig. 4). The interaction of *M. tuberculosis* of *WhiB1* with σ^A depends on the presence of the *WhiB1* [4Fe-4S] cluster [Kudhair et al., 2017; Wan et al., 2020]. In *M. tuberculosis*, $\sigma^{A_{CTD}}$ inserts His516 and Pro517 of the C-terminal loop into the binding pocket of the *WhiB1* [4Fe-4S] cluster to initiate the interaction, while in *WhiB1*, the binding pocket is a cluster of hydrophobic residues including Trp3, Phe17, Trp60, and Phe18, which are highly conserved in *WhiB1* cross actinobacteria [Wan et al., 2020]; those residues are also conserved in *K. sedentarius* MBB13 *WhiB1* (online supplementary Fig. 3). Therefore, the mechanism of *WhiB1*: $\sigma^{A_{CTD}}$ interaction is likely to be conserved in *K. sedentarius* MBB13 and *M. tuberculosis*.

Nitric oxide reacted with the [4Fe-4S] clusters of the *WhiB1* and *WhiB2* proteins (Fig. 5) and the BACTH inter-

actions were sensitive to conditions that generated NO, suggesting that *K. sedentarius* MBB13 *WhiB1* and *WhiB2* alter σ^A function in response to nitric oxide. Strains of *K. sedentarius* that possess a nitrate reductase could be exposed to endogenous nitric oxide, generated by nitrite reduction. However, *K. sedentarius* MBB13 lacks a nitrate reductase and hence *WhiB1* is more likely to respond to exogenous nitric oxide, perhaps generated by other microbes or the host immune system. Furthermore, the iron-sulfur cluster of *K. sedentarius* MBB13 *WhiB2* appeared to be oxygen-sensitive and so could act as a sensor of oxygen availability, an important parameter for an aerobe, rather than nitric oxide. Further research will be needed to test this speculation and addresses the open questions, including: why are multiple Wbl proteins present, how do *WhiB1* and *WhiB2* (and possibly *WhiB3*) alter gene expression though their interactions with σ^A , and what is the primary signal(s) that cause disassembly of the Wbl- σ^A complexes?

Acknowledgments

We thank Professor Milton Wainwright (University of Sheffield) for initiating the project, and Dr Joseph Webb and Dr James Gilmour (University of Sheffield) for useful discussions and advice on experimental design and Dr Andrea Hounslow (University of Sheffield) for assistance with NMR experiments, and Professor David Kelly (University of Sheffield) for access to laboratory equipment.

Statement of Ethics

The authors declare that the experiments reported were compliant with the regulations overseen by the University of Sheffield Biosafety Committee under the following reference numbers: GMO1984_01 and GMO2010_03A.

Conflict of Interest Statement

The authors declare that they have no competing interests.

Funding Sources

This work was supported by the Royal Embassy of Saudi Arabia Cultural Bureau in London and Saudi Food and Drug Authority (SFDA) through a PhD scholarship to M.A.A.

Author Contributions

J.G. and M.A.A. conceived the study. M.A.A., J.G. and B.K.K. performed the experiments. M.A.A. and J.G. drafted the manuscript. All authors read and approved the final manuscript.

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