Transcriptional cross-regulation as survival mechanism in bacteria

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Abstract

The high number of metal resistance genes in the soil bacterium *Cupriavidus metallidurans* CH34 makes it an interesting model organism to study microbial heavy metal responses. A first step in understanding the molecular mechanisms that underlie heavy metal resistance is to reconstruct the transcriptional regulatory networks. Therefore genomewide expression experiments were performed to investigate the full stress response of *C. metallidurans* CH34 when it was challenged to a variety of heavy metals including zinc, copper, cadmium, and lead. Certain heavy metal response gene clusters showed similar expression profiles when cells were found to be exposed to varying combinations of heavy metals, thus pointing to complex cross-talk at the transcriptional level between the different heavy metal resistance mechanisms. Our results could partially explain this cross-talk by identification and similarity analysis of transcription factor binding sites in the promoter region of metal resistance genes.

This hypothesis is further confirmed by a directed evolution experiment, exposing the bacterium to toxic concentrations of silver, resulting in mutants with an increased resistance towards this metal. Subsequent Illumina sequencing of two of these mutant strains points towards an inactivation of the sensory component of the two-component regulatory system AgrR/S. Remarkably, this regulatory system belongs to a region predicted based on homology to be important for metal resistance (*agrRSCBA*), however without ever being expressed in any transcriptomic study investigating metal resistance. A phylogenetic footprinting approach of the *agrR* promoter region predicts a regulatory motif which resembles the regulatory motifs found in other identified metal resistance regions. This way, the AgrR transcription factor would not only activate its own dedicated metal efflux pump AgrCBA, but also other metal resistance regions containing a similar transcription factor binding site in their promoter region, which indeed could be confirmed by gene expression analysis.

Introduction

The β-proteobacterium *Cupriavidus metallidurans* was isolated in 1976 as a cadmium-resistant and hydrogen-oxidizing pseudomonad from a decantation tank at a metal processing factory in Belgium. This strain, later called CH34, was shown to tolerate high concentrations of metals like copper, zinc,

nickel, cadmium and lead. Recent sequencing and annotation efforts lead to the identification of two chromosomes and two large plasmids, where all four replicons contain essential metal resistance regions, often located on mobile genetic elements [1, 2]. In this study, transcriptomic analyses were performed in order to identify the genetic determinants responsible for heavy metal resistance in *C. metallidurans* CH34. As this study revealed that a complex interplay existed at transcriptional level between different metal resistance mechanisms, regulatory motif searches were performed to decipher the regulatory network underlying this complex interplay. Additionally, a directed evolution experiment leading to mutants with an increased metal resistance further underpins this hypothesis of cross-regulation. Via full genomic sequencing and expression analysis, cross-regulation was identified as one of the key mechanisms leading to an increased metal resistance of these mutants.

Results & Discussion

Transcriptional cross-regulation between metal resistance mechanisms

In order to measure the transcriptional response of *C. metallidurans* CH34 after metal exposure, microarray experiments were performed exposing the bacterium to 16 different metals at sublethal concentration. The expression data where then used to identify potential metal resistance clusters by looking for neighboring genes which were upregulated by one or more metals, resulting in a total of 36 potential metal resistance regions. The mosaic of metal response clusters used by multiple metals in different combinations highlights the complex transcriptional response in *C. metallidurans* CH34, where one metal resistance region can be switched on by multiple metals, and reversely one metal most often transcriptionally induces different metal resistance clusters.

Phylogenetic footprinting shows similarities in transcription factor binding sites

A phylogenetic footprinting approach was applied to the orthologous promoter regions of all regulatory proteins potentially involved in metal resistance i.e. regulatory proteins of gene clusters being differentially expressed in one of the microarray studies, or clusters predicted to be important for metal resistance based on homology searches. The reasoning behind this approach is the fact that an important part of bacterial two-component regulatory systems are subjected to autoregulation, implying that the binding site of the transcription factor itself needs to be present in its own promoter region. The highly redundant nature of these heavy metal response gene clusters forced us to use a strict definition of orthologs being reciprocal best blast hits. By combining the output of four different motif detection algorithms (GLAM [3], AlignACE [4], MEME [5] and MotifSampler [6]) in an ensemble-like way,

regulatory motifs could be found for the majority of putative regulators of metal resistance proteins. From the total of 28 regulatory proteins subjected to this approach, a regulatory motif was found for 23 of them. Subsequent comparison between the different motif models showed a significant similarity between the transcription factor binding sites of different metal resistance clusters, possibly explaining the transcriptional cross-regulation observed between them.

Directed evolution reveals mutants with increased metal resistance

Where previous indications above on transcriptional cross-regulation where mainly based on in silico prediction of regulatory motifs, a more biologically relevant approach is provided by a directed evolution experiment. Two independent mutants of *C. metallidurans* CH34 were isolated after prolonged incubation on rich medium supplemented with $AgNO_3$. These mutants (AE2720 and AE2722) showed an increased resistance to silver compared to the wild-type CH34 (up to 20 times higher). Subsequent microarray analysis comparing both mutants with the wild type strain in absence and presence of silver respectively, showed a differential expression of hundreds of genes with a very high overlap in expressed genes between both mutants (overlap of around 90% of differentially expressed genes). Remarkably, a high number of metal response regions – identified as described in the first paragraph – were also transcriptionally activated in both mutants e.g. the *czc* region with resistance determinants towards cobalt, zinc and nickel, and the *cop* regulon partially responsible for copper resistance. However, from the three systems (*cus, sil, cup)* known to be important for silver resistance in strain CH34, none was differentially expressed. Full genome sequencing – using a 50 bp paired end approach with 300 bp insert size on the Illumina GAIIx platform – was used to identify the mutations responsible for the increases silver resistance. Apart from some point mutations and insertions and deletions of mobile genetic elements at various locations in both strains, the only common mutation observed in both silver resistant strains was the translocation of insertion sequence ISRme3 from its original location into the coding sequence of a signal transduction histidine kinase *agrS*. Remarkably, the two-component regulatory system AgrRS is associated to the *agrCBA* operon encoding for a RND metal efflux transporter. This region was already predicted based on homology to be important for silver resistance, but was not found to be expressed in any of the metal induction experiments. Since the operon *agrCBA* is up-regulated in only one of the two mutants, its role in the increased silver resistance is ambiguous. Therefore, the AgrRS system would rather play its role in increasing the silver resistance by cross-regulating other two-component regulatory systems, which might explain the significant changes in gene expression profiles.

Cross-regulation as powerful survival tool

Focusing on the regulatory motif detected based on phylogenetic footprinting in the upstream region of the *agrRS* operon, a clear similarity is observed with the putative transcription factor binding sites as observed in other metal resistance regions like *czc* (responsible for cobalt, cadmium and zinc resistance) and the chromosomal and plasmid borne *cop* (responsible for copper resistance) cluster. This observation further underpins the hypothesis that the mutation in the AgrRS two-component system activates other stress-related regions via transcriptional cross-regulation. Additionally, using the regulatory motif model upstream of *agrRS* for a genomewide screening of all upstream regions in *C. metallidurans* CH34, an AgrR binding site is revealed in other metal related genes like *mmmQ* and *mmsQ*, and which were also found to be differentially expressed in the mutant strains compared with the wild type.

Conclusion

Based on genomewide expression data we could identify genetic determinants responsible for metal resistance. Moreover, the interaction observed between the different metal response clusters could be explained by transcriptional cross-talk due to similarity in transcription factor binding sites. Using a directed evolution experiment resulting in mutants with an increased silver resistance, it was shown that an insertion of a mobile genetic element into a two-component system leads via transcriptional cross-talk to an activation of other metal resistance clusters. As such, this transcriptional cross-talk seems to be an essential survival mechanism for *C. metallidurans* CH34.

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