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Chlorate reductase is cotranscribed with cytochrome c and other downstream genes in the gene cluster for chlorate respiration of *Ideonella dechloratans*

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One sentence summary: Chlorate reductase of the chlorate degrading bacteria *Ideonella dechloratans* is cotranscribed with genes that may function in electron transfer, cofactor biosynthesis and gene regulation.

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ABSTRACT

The chlorate-respiring bacterium *Ideonella dechloratans* is a facultative anaerobe that can use both oxygen and chlorate as terminal electron acceptors. The genes for the enzymes chlorate reductase (*clrABDC*) and chlorite dismutase, necessary for chlorate metabolism and probably acquired by lateral gene transfer, are located in a gene cluster that also includes other genes potentially important for chlorate metabolism. Among those are a gene for cytochrome c (*cyc*) whose gene product may serve as an electron carrier during chlorate reduction, a cofactor biosynthesis gene (*mobB*) and a predicted transcriptional regulator (*arsR*). Only chlorate reductase and chlorite dismutase have been shown to be expressed in *vivo*. Here, we report the *in vivo* production of a single polycistronic transcript covering eight open reading frames including *clrABDC, cyc, mobB* and *arsR*. Transcription levels of the *cyc* and *clrA* genes were compared to each other by the use of qRT-PCR in RNA preparations from cells grown under aerobic or chlorate reducing anaerobic conditions. The two genes showed the same mRNA levels under both growth regimes, indicating that no transcription termination occurs between them. Higher transcription levels were observed at growth without external oxygen supply. Implications for electron pathway integration following lateral gene transfer are discussed.

Key words: oxochlorates; anaerobic respiration; gene expression

INTRODUCTION

Chlorate (*ClO₃⁻*) and perchlorate (*ClO₄⁻*) are toxic compounds, mostly of anthropogenic origin, which have been released into soils and waters during the last century (Logan 1998). They are now recognized as environmental hazards, resulting in a need for dealing with oxochlorate contamination. For both compounds, bioremediation techniques based on microbial degradation by anaerobic respiration are used (Logan 1998; Coates and Achenbach 2004). Dissimilatory chlorate- and perchlorate-reducing bacteria, most of which are facultative anaerobes of the Proteobacteria group, seems to be widely distributed in Nature (Coates et al. 1999; Bardiya and Bae 2011), probably due to deposition of natural chlorate and perchlorate of atmospheric origin (Kounaves et al. 2010; Rao et al. 2010). Reduction of perchlorate and/or chlorate is catalyzed by either a (per)chlorate reductase (Pcr) in (per)chlorate-reducing bacteria (PRB) which are able to perform the sequential reduction of perchlorate to chlorate and chlorate to chlorite (Kengen et al. 1999), or a chlorate reductase (Clr) in chlorate-reducing bacteria (CRB) which only reduces chlorate to chlorite (Danielsson Thorell et al. 2003; Wolterink et al. 2003). Both CRB and PRB use the enzyme chlorite dismutase...
(Cld) to decompose the toxic chlorite to chloride ions and molecular oxygen. The produced oxygen is assumed to be utilized by a terminal oxidase (Rikken, Kroon and van Ginkel 1996). While the three key enzymes have been isolated and characterized in several strains (Nilsson, Rova and Smedje Bäcklund 2013), not much is known about the other components necessary for dissimilatory chlorate or perchlorate reduction, such as the participants of the electron transfer chains or regulatory factors.

*Ideonella dechloratans* (Malmqvist et al. 1994) was the first CRB to have its genes for chlorite dismutase (cld) and chlorate reductase (clrABDC) sequenced and these genes were found to be arranged in a gene cluster which later was shown to also include a cytochrome c gene, at first denoted ctc, here designated ctc (GenBank EU768872.1) and a molybdopterin-guanine dinucleotide biosynthesis gene (mobB) (Danielsson Thorell et al. 2002; Danielsson Thorell et al. 2003; Bohlin et al. 2010). Clr is a periplasmic molybdoenzyme of the type II DMSO reductase family (Rothery, Workun and Weiner 2008) with a subunit stoichiometry of α, β, γ1, encoded by the clrABDC operon (Danielsson Thorell et al. 2003). The cld gene is located upstream of clrABDC, orientated in the opposite direction and separated from ctc by an insertion element (ISId1). The ctc gene, which encodes a soluble c-type cytochrome, has the same direction as clrABDC and is found 126 bp downstream of ctc (Bohlin et al. 2010). A similar arrangement of the cld and clrABDC genes was later established in *Pseudomonas chloritidismutans* AW-1 (GenBank GG919187.1). Moreover, a plasmid-borne sequence almost identical to the cld gene was found in the benzene-degrading CRB *Acyliciphilus denitrificans* BC (Oosterkamp et al. 2011).

Both the cpr and the PRB are phylogenetic diverse groups which has led to the suggestion that chlorate and (per)chlorate metabolism can be acquired by horizontal gene transfer. Recently, more evidence of this has come from sequence analyses (Melnyk et al. 2011; Clark et al. 2013). In four (per)chlorate-reducing strains, the cld gene and the genes for (per)chlorate reductase (pcrABCD) were found to form clusters together with suggested accessory genes in areas identified as genomic islands that show signs of having been mobilized and integrated into the host (Melnyk et al. 2011). New sequence information from Clark et al. (2013) adds three more CRBs with similar arrangement of the cld and clrABDC genes as the three strains described earlier. Analyses of these six chlorate clusters resulted in identification of insertion sequences flanking five of them, thus forming composite transposons some of which may have the potential to move horizontally. In I. dechloratans, these insertion sequences were reported to be 99% identical to ISAav1 from *Acidovorax citrulli* AAC001–1. Eight open reading frames (ORFs) arranged in one direction: ctdABCD, ctc, mobB, arsR (predicted to encode a transcriptional regulator of the ArsR family) and a hypothetical protein were found in a region delimited by ISId1 and one of the ISAav1 like insertion sequences (Clark et al. 2013) (Fig. 1).

An interesting question is how respiratory components acquired by lateral gene transfer are integrated with the pre-existing electron transport chain of the host. For dissimilatory chlorate and (per)chlorate reduction, the soluble oxidoreductase has to be connected to the quinone pool of the bacteria’s inner membrane. Different proposals of how this is arranged have been made for the two groups; interaction between Pcr and a membrane-associated c-type cytochrome of the NapC/NrfH family was predicted for the PRB *Dechloromonas agitata* and *D. aromatica* (Bender et al. 2005) whereas a soluble c-type cytochrome connecting a membrane-bound cytochrome bc; quinol:cytochrome c oxidoreductase with Clr was suggested for *I. dechloratans* (Bäcklund and Nilsson 2011). Notably, all four (per)chlorate reduction-associated genomic islands contain a gene predicted to encode a membrane-associated c-type cytochrome with quinol dehydrogenase activity (Melnyk et al. 2011), and the five composite transposons of the CRB all contain a gene predicted to encode a soluble c-type cytochrome (Clark et al. 2013), indicative of different electron pathways in the two respiratory metabolisms.

The cyc gene of the chlorate cluster in *I. dechloratans*, which is not encoding cty c-I1, has been cloned and heterologously expressed in *Escherichia coli* where it translated into a protein that after reconstitution with heme showed a spectrum typical for a c-type cytochrome, indicating functionality (Bohlin et al. 2010). However, the reconstituted protein could not be shown to donate electrons to Clr in vitro (Bohlin et al. 2010) and the search for the gene product in periplasma preparations of *I. dechloratans* has hitherto been unsuccessful. This raises the question if the function of this gene has been taken over by the chromosomally encoded cty c-I1 as an example of evolutionary integration, if it has a yet undiscovered function, or if it was just accidentally transferred in the proposed transposition event. We have performed transcriptional studies by the use of qRT-PCR and reverse transcription PCR on RNA preparations of *I. dechloratans* grown under aerobic and anaerobic, chlorate reducing, conditions in order to contribute to the answer to this question.

**MATERIALS AND METHODS**

**Bacterial growth conditions**

*I. dechloratans* was obtained from the culture collection of Göteborg University, Göteborg, Sweden (CCUG 30977). Overnight
cultures were grown aerobically in 5 ml PC medium (5 g tryptone and 2.5 g yeast extract per liter deionized water, pH 7.0), 37 °C. Thereafter, cells were cultivated under two different growth conditions: (i) aerobically in PC medium with oxygen as sole electron acceptor, or (ii) anaerobically in a mineral medium with acetate (25 mM) as the electron donor and carbon source, and sodium chlorate (10 mM) as the electron acceptor, as previously described (Hellberg Lindqvist et al. 2012). Cell cultures were harvested in mid-log phase measured as OD600 of 0.4 to 0.6. Aerobic cultures were grown 3–4 h and the anaerobic cultures 20–24 h after subcultivation to reach mid-log phase.

RNA preparation and cDNA synthesis
Preparation of total RNA and cDNA synthesis with random hexamer primers was performed as described in (Hellberg Lindqvist et al. 2012). The specific cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer using 1 μM reverse primer arsR-hypothetical protein (Table S1, Supporting Information) instead of the random hexamer primers included in the kit. 1 μg of DNase-treated total RNA from I. dechloratans cultivated under anaerobic conditions was added and the final volume was adjusted to 20 μl. Two types of negative controls were used in all cDNA synthesis reactions: (1) reverse transcriptase-negative control containing all components of the cDNA synthesis except for reverse transcriptase and (2) no-template control (NTC) with water instead of RNA.

Primer design
PCR primers for the genes clrA, clrB, clrD and clrC (GenBank AJ566363.1, cyc and mobB (GenBank EU768872.1), arsR (IMG GeneID 2510554292), a hypothetic protein (IMG GeneID 2510554293) and 16S rRNA (GenBank X72724.1) were designed by using Primer-BLAST (NCBI) or the PrimerSelect application of Lasergene (DNASTar). The PCR reactions were analyzed with agarose gel electrophoresis and the expected product sizes could be verified as single bands (data not shown). Primers and expected product sizes are listed in Table S1 (Supporting Information).

Real-time qRT-PCR
Real-time qRT-PCR was performed on a Step One Plus instrument (Applied Biosystems) using Fast SYBR® Green Master Mix (Applied Biosystems) as previously described (Hellberg Lindqvist et al. 2012). Final concentrations of gene-specific primers were between 0.2-0.3 μM. Each sample was measured in triplicate. Amplification efficiency was determined using a 10⁴- to 10⁸-fold range of template cDNA concentrations in triplicate with the specific primers for 16S rRNA, cyc and clrA. The slope of the PCR amplification thus obtained for the genes 16S rRNA, cyc and clrA varied between −3.46 and −3.57 (R² value > 0.99). The corresponding efficiency (E) of the qRT-PCR (Table S2, Supporting Information) was calculated according to the equation: $E = \left(10^{(-\text{slope})} - 1\right) \times 100$, where an efficiency of 100% represents a PCR reaction that doubles the amount of product in each cycle. The $2^{-\Delta \Delta C_T}$ method was applied to calculate the fold difference of the relative expression levels for two different genes (target A and B) or for the same gene at two different growth conditions (target A and B) and 16S rRNA was used as reference gene (Eqs 1 and 2).

\[ \Delta \Delta C_T = (C_T^{\text{target A}} - C_T^{\text{reference A}}) - (C_T^{\text{target B}} - C_T^{\text{reference B}}) \] (1)

\[ \text{Fold difference of relative expression levels} = 2^{-\Delta \Delta C_T} \] (2)

A primer optimization matrix was setup for the primer pairs for 16S rRNA and cyc following the recommendations by (Nolan, Hands and Bustin 2006). Four different primer concentrations, 50, 100, 200 and 300 nM, and their combinations were investigated. Primer concentrations used in the qRT-PCR were selected according to low Ct value in combination with high end-point fluorescence (ΔR) in the absence of primer dimers.

PCR
The primer pairs constructed for chrA-clrB, chrB-clrD, chrD-chrC, chrC-cyc, cyc-mobB, mob-arsR and arsR-hypothetic protein (Table S1, Supporting Information) were found to give PCR products of expected sizes (Fig. 2). PCR reactions were set up with both genomic DNA (gDNA) (3 ng) and cDNA (80 ng) as template. For the PCR reaction chrC-cyc, a HotStar Taq® Plus Master Mix Kit (Qiagen) was used with a final primer concentration of 0.3 μM. PCR program used was; initial step of 95 °C 5 min, followed by 35 cycles of 94 °C 1 min, 55 °C 1 min and final annealing step of 72 °C 20 min. For the additional PCR reactions, a KAPA2G Robust HotStart ReadyMix (KapaBiosystems) was used with a final primer concentration of 0.5 μM. PCR program used was; initial step of 95 °C 1 min, followed by 35 cycles of 94 °C 15 s, 55 °C 15 s, 72 °C 15 s and final annealing step of 72 °C 10 min. The two negative controls from the cDNA synthesis were run in all the PCR reactions using the same primers as above along with a NTC of the PCR reactions containing all components except for template.

An additional PCR reaction with the product from the specific cDNA (80 ng) synthesis was set up with the primer pairs chrA-clrB, chrD-clrC and arsR-hypothetic protein. A KAPA2G Robust HotStart ReadyMix (KapaBiosystems) was used with the same PCR conditions and negative controls as previous PCR reactions.

DNA Sequencing
The PCR products with cDNA as template was subcloned into the T-tailed vector pGEM®-T Easy (Promega) by 1 h ligation at RT or overnight ligation at 8 °C using T4 DNA ligase (Fermentas). Chemically competent E. coli XL-1 Blue cells were transformed with the pGEM®-T constructs. Positive colonies were cultivated overnight in LB medium with ampicillin 100 μg ml⁻¹ followed by plasmid preparation with a Plasmid DNA Mini Kit I (Omega) using the Plasmid Mini Spin Protocol. Cycle sequencing reactions were set up using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), following the recommendations of the manufacturer. The sequencing was then performed on a 3130xl Genetic Analyzer (Applied Biosystems) by the Department of Molecular Biology, Umeå University, Sweden.

RESULTS AND DISCUSSION
qRT-PCR was undertaken to determine if the cyc gene of the chlorate cluster is transcriptionally active. cyc-specific mRNA was detected in samples from cells grown under anaerobic chlorate reducing as well as aerobic conditions (Table 1), showing that the gene is transcribed under both growth regimes. Normalized mRNA levels were found to be about 9 times higher in
Table 1. Relative mRNA levels of clrA and cyc

<table>
<thead>
<tr>
<th>Condition</th>
<th>ΔCt (cyc)</th>
<th>ΔCt (clrA)</th>
<th>2−ΔΔCt (clrA:cyc)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td>12.59</td>
<td>12.40</td>
<td>1.16 ± 0.10</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td>9.63</td>
<td>9.65</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>(anaerobic:aerobic)</td>
<td>9.21 ± 2.62</td>
<td>7.99 ± 2.17</td>
<td></td>
</tr>
</tbody>
</table>

ánio: calculated by subtracting the Ct value of the reference gene (16S rRNA) from the Ct value of the target gene (cyc or clrA).

<table>
<thead>
<tr>
<th>Fold difference of the relative mRNA levels between the genes clrA and cyc, shown as mean value ± SEM (N = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(anaerobic:aerobic)</td>
</tr>
</tbody>
</table>

| ΔCt was calculated by subtracting the Ct value of the reference gene (16S rRNA) from the Ct value of the target gene (cyc or clrA). |
|ΔΔCt was calculated by subtracting the ΔCt of the target gene from the ΔCt of the reference gene. |
|Fold change between anaerobic and aerobic relative mRNA levels of the genes cyc and clrA respectively, shown as mean value ± SEM (N = 5) |

The presence of a joint transcript between one of the key enzymes for chlorate reduction, Clr, and downstream genes with potential functions in electron transfer, cofactor biosynthesis, and gene regulation may indicate an importance, present or historic, of these downstream genes in chlorate reduction rather than being accidental passengers in a transposition event. Although a candidate hairpin-type transcription terminator is predicted in the intergenic region between clrC and cyc by some analysis services, our finding that the cyc gene is transcribed at the same level as clrA indicates that no transcription termination occurs between those genes. When expressed in E. coli, the cyc gene seems to translate into a functional c-type cytochrome (Bohlin et al. 2010). The protein is predicted to have a signal peptide and to lack transmembrane helices and would therefore be expected to be soluble in the periplasm, if translated in I. dechloratans. Despite efforts to identify the gene product of cyc, no corresponding protein has been found in I. dechloratans. However, it cannot be ruled out that the protein has escaped
et al. 2009). Of those, only the 6 kDa protein, later labeled cytochrome c-Id1, has been shown to serve as electron donor for chlorate reduction (Bäcklund and Nilsson 2011). The presence of another electron donor to chlorate reductase questions a present role of cyc in chlorate respiration. However, it is possible that the cyc gene, if translated, have a function in chlorate reduction together with cytochrome c-Id1. Cytochrome c-Id1 was shown to have a dual role in the electron transport chain as it is able to donate electrons to a terminal cytochrome c oxidase in addition to Clr (Bäcklund et al. 2009). Electron supply to Clr by two pathways may be beneficial or just reflect a gradual integration of chlorate metabolism into the host. Interestingly, redundancy in the electron transport chain to Pcr was recently suggested for the PRB Azospira suillum PS based on the observation that deletion of the quinol dehydrogenase of the perchlorate genomic island resulted in only partially impaired growth on perchlorate, indicating the presence of additional electron pathways (Melnyk et al. 2014).

In conclusion, we have detected the production of a single polycistrionic mRNA from the chlorate metabolism gene cluster in I. dechloratans, including the cyc, mobB and arsR genes in addition to clrABDC. The cyc gene appears to be regulated at the transcriptional level by an oxygen- or redox-dependent mechanism similar to that of clrABDC. From these results, one would expect the Cyc protein to be produced and due to the presence of other cytochromes in the periplasm, one would also expect the availability of a functional machinery for heme export and insertion (Sanders et al. 2010). The presence of the gene product at the protein level remains, however, to be demonstrated for cyc as well as for mobB and arsR.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

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Conflict of interest statement. None declared.

REFERENCES


