ORIGINAL ARTICLE

Correlating carbon monoxide oxidation with *cox* genes in the abundant Marine Roseobacter Clade

Michael Cunliffe

Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth, UK

The Marine Roseobacter Clade (MRC) is a numerically and biogeochemically significant component of the bacterioplankton. Annotation of multiple MRC genomes has revealed that an abundance of carbon monoxide dehydrogenase (CODH) *cox* genes are present, subsequently implying a role for the MRC in marine CO cycling. The *cox* genes fall into two distinct forms based on sequence analysis of the *coxL* gene; forms I and II. The two forms are unevenly distributed across the MRC genomes. Most (18/29) of the MRC genomes contain only the putative form II *coxL* gene. Only 10 of the 29 MRC genomes analysed have both the putative form II and the definitive form I *coxL*. None have only the form I *coxL*. Genes previously shown to be required for post-translational maturation of the form I CODH enzyme are absent from the MRC genomes containing only form II. Subsequent analyses of a subset of nine MRC strains revealed that only MRC strains with both *coxL* forms are able to oxidise CO.

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Introduction

The availability of numerous bacterial genomes has provided the opportunity to make pan-genomic comparisons between taxa. A bacterial taxon which has received considerable effort in terms of genome sequencing is the Marine Roseobacter Clade (MRC) (Moran *et al.*, 2007; Newton *et al.*, 2010). The MRC is a phylogenetically coherent group in the order *Rhodobacterales* and is one of the most numerically abundant marine lineages, especially in coastal and polar waters, accounting for up to 25% of the bacterioplankton (Giovannoni and Rappé, 2000; Wagner-Döbler and Biebl, 2006; Brinkhoff *et al.*, 2008).

The MRC is a physiological diverse group that has been implicated in a range of biogeochemical processes (Moran and Miller, 2007; Moran *et al.*, 2007; Newton *et al.*, 2010). These include the degradation of algal-derived sulfur compounds, such as dimethylsulfoniopropionate, (González *et al.*, 1999), aerobic anoxygenic photosynthesis (Yurkov and Beatty, 1998), methyl halide metabolism (Schafer *et al.*, 2005) and the oxidation of manganese (Hansel and Francis, 2006). The annotation of the genome of *Ruegeria pomeroyi* DSS-3

(basonym *Silicibacter pomeroyi*) lead to the discovery of carbon monoxide dehydrogenase (CODH) genes, suggesting yet another biogeochemical role for the MRC (Moran *et al.*, 2004).

The *R. pomeroyi* DSS-3 genome has two distinct CO oxidation gene clusters, both of which contain different *coxL* genes (Moran *et al.*, 2004). The *coxL* gene encodes the large catalytic subunit of CODH and the divergence of the two genes has led to two gene clusters being identified; form I, also known as the OMP group (from *Oligotropha*, *Mycobacterium* and *Pseudomonas*) and form II, also known as the BMS group (from *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium*) (King, 2003). The form I CODH constitutes the definitive CODH and is well characterised in the 'classic' carboxydrotrophs *Oligotropha carboxidovorans* and *Pseudomonas carboxydohydrogena* (Meyer and Schlegel, 1983; King and Weber, 2007). The form II CODH is a putative CODH and its function is largely inferred from sequence homology with the form I *coxL* gene (King, 2003; King and Weber, 2007). There is some evidence that the form II CODH has a lower affinity for CO compared with the form I CODH (Lorite *et al.*, 2000), and this has led to the hypothesis that the presence of both forms of CODH offers an ecological advantage under a range of CO concentrations (King, 2003; King and Weber, 2007). King and Weber (2007) have also suggested that, as the form II CODH does not have the characteristic AYXCSFR motif found in the form I CODH, the enzyme's primary function may not be CO oxidation, and CO oxidation is a secondary or incidental reaction.

Correspondence: M Cunliffe, Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK.

E-mail: micnli@mba.ac.uk

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Since the discovery of *cox* genes in the genome of *R. pomeroyi* DSS-3, more MRC genomes have been sequenced. Moran *et al.* (2007) compared 12 MRC genomes, including *R. pomeroyi* DSS-3, and identified at least one *cox* gene cluster in all but one genome. More recently, Newton *et al.* (2010) performed a comprehensive pan-genome assessment of 32 MRC genomes, which again showed the prevalence of *cox* genes.

The objectives of this study are to characterise the distribution of *cox* genes in the available MRC genomes, to conduct a comparative analysis of the two forms of *coxL* with associated *cox* genes and to establish how widespread CO oxidation capability is across the MRC by correlating CO oxidation with the different CODH forms.

Materials and methods

The genome sequences of 29 MRC strains (Table 1 and Figure 1) were studied using the RoseoBase online genomic resource for marine Roseobacters (www.roseobase.org). The characteristics of each of the MRC strain genomes used in this study have been reported in detail previously by Newton *et al.* (2010). *coxL* genes were identified in each genome sequence by using the blastx algorithm (that is, search the protein database using a translated nucleotide query). The *coxL* genes from *R. pomeroyi* DSS-3 were blasted sequentially against each genome and homologues were selected for analysis.

The collated *coxL* gene sequences were imported into a MEGA database (Tamura *et al.*, 2007), translated *in silico* and the amino acid sequences aligned. The subsequent phylogenetic dendrograms produced from the amino acid alignments were constructed using a neighbour-joining algorithm with a Poisson correction model and 1000 bootstrap replications. The 16S rRNA gene sequences from the MRC strains were also imported and aligned using MEGA. For the 16S rRNA gene dendrogram, a neighbour-joining algorithm, with a Maximum Composite Likelihood model and 1000 bootstrap replications, was used.

Genes neighbouring the identified *coxL* genes in the genome were also considered. There was some inconsistency in the extent of genome annotation between the MRC strain genomes used in this study at the time of analysis (February 2010). Therefore, all flanking genes were manually checked for homology with other *cox* genes using blast (Altschul *et al.*, 1990) via the National Centre for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/>).

Nine MRC strains were selected to determine CO oxidation capability (Table 1) and were chosen to span the MRC phylogenetic tree (Figure 1). *Roseovarius* sp. 217 was a gift from Dr Hendrik Schäfer at the University of Warwick (UK). All the other strains were purchased from the Deutsche

Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The strains were maintained in the laboratory on marine agar 2216 (Difco, BD, NJ, USA). To determine CO oxidation, strains were grown in 50 ml marine broth 2216 (Difco) in triplicate 250 ml Erlenmeyer flasks fitted with a Suba-Seal (Sigma Aldrich, Gillingham, UK). Carbon monoxide was added to the flask headspaces (1000 p.p.m.), and the strains were incubated with shaking (150 r.p.m.) at 30 °C, except for *R. denitrificans* and *R. littoralis*, which were incubated at 20 °C. Carbon monoxide uptake was monitored at intervals using an electrochemical CO metre (Anton, Watford, UK) and growth was determined using final absorbance (OD at 600 nm).

Results and discussion

As reported previously by Moran *et al.* (2007) and Newton *et al.* (2010), *cox* genes are prevalent in MRC genomes. In this study, at least one *coxL* form was identified in 28 of the 29 MRC genomes analysed (Table 1). Only *Roseovarius nubinhibens* ISM had no detectable *coxL* genes. In this study, a *coxL* form II homologue was detected in the genome of *Oceanicola granulosus* HTCC 2516, the previous study by Newton *et al.* (2010) reported no *cox* genes in this organism. The putative *O. granulosus* HTCC 2516 *coxL* form II homologue is divergent from the other MRC *coxL* gene sequences (Figure 2), but analysis of the translated amino acid sequence of *O. granulosus* HTCC 2516 shows that the AYRGAGR motif is present (data not shown), which characterises the form II CODH (King, 2003). *cox* genes have proven to be abundant in environmental samples. Metagenomic analyses of the Sargasso Sea (Moran *et al.*, 2004; King and Weber, 2007) revealed that a significant number of *cox* genes are present, with some samples showing as many as 1 in 10 bacterial cells contain a *coxL* gene (Moran *et al.*, 2004).

The distribution of the two *coxL* gene forms was markedly different across the MRC genomes (Table 1). Both *coxL* gene forms were detected in 10 MRC genomes and only the form II *coxL* was detected in 18 MRC genomes. No genomes had only the form I *coxL* detected, which encodes the large subunit of the definitive CODH (King, 2003). MRC strains, which had both the gene forms were not monophyletic, but spread across the whole of the MRC 16S rRNA gene phylogenetic tree, with closely related strains having only the form II *coxL* (Figure 1). The distribution of both *coxL* forms in relation to isolation source (that is, free-living or attached/associated) was analysed using a contingency table and chi-squared (χ^2) test. The test showed no evidence ($\chi^2 = 0.189$; χ^2 must be > 3.84 for a 5% significance level) of dependency of *coxL* gene distribution on the two variables.

Phylogenetic analysis of the MRC *coxL* genes illustrates clearly the two forms of the gene

Table 1 Distribution of *coxL* genes and carbon monoxide oxidation in the Marine Roseobacter Clade strains compared in this study

Organism	Strain	Isolated from ^a	coxL form		CO oxidation
			I	II	
<i>Phaeobacter gallaeciensis</i>	2.10	Surface of macroalgae <i>Ulva lactuca</i> . Sydney, Australia	ND	Yes	
<i>Phaeobacter gallaeciensis</i>	BS107	Scallop larvae <i>Pecten maximus</i>	ND	Yes	–
<i>Roseobacter</i> sp.	MED193	North-west Mediterranean sea (1 m depth)	Yes	Yes	
<i>Roseobacter</i> sp.	SK209-2-6	Oxygen minimum zone (267 m depth). Arabian sea	Yes	Yes	
<i>Ruegeria</i> sp.	R11	Surface of the macroalgae <i>Delisea pulchra</i> . Bare Island, Sydney, Australia	ND	Yes	
<i>Ruegeria pomeroyi</i>	DSS-3	Enriched for growth on dimethylsulfoniopropionate. Coast of Georgia, USA	Yes	Yes	+
<i>Ruegeria</i> sp.	TM1040	Phycosphere of dinoflagellate <i>Pfiesteria piscicida</i>	ND	Yes	
<i>Pelagibaca bermudensis</i> ^b	HTCC2601	Bermuda Atlantic time series study station (10 m depth)	ND	Yes	
<i>Roseobacter</i> sp.	AzwK-3b	Estuary adjacent to Monterey Bay, California, USA	ND	Yes	
<i>Roseovarius nubinhibens</i>	ISM	Caribbean sea (22°4'N, 74°4'W)	ND	ND	
<i>Roseovarius</i> sp.	217	Western channel observatory, L4 station. English Channel, UK	Yes	Yes	+
<i>Roseovarius</i> sp.	TM1035	Dinoflagellate culture <i>Pfiesteria piscicida</i>	Yes	Yes	
<i>Dinoroseobacter shibae</i>	DFL 12	Dinoflagellate culture <i>Prorocentrum lima</i>	Yes	Yes	+
<i>Maritimibacter alkaliphilus</i>	HTCC2654	Bermuda Atlantic time series study station (10 m depth)	ND	Yes	
<i>Sagittula stellata</i>	E-37	Enriched for growth on lignocellulose. Coast of Georgia, USA	ND	Yes	–
<i>Jannaschia</i> sp.	CCS1	Bodega Head, California, USA	Yes	Yes	
<i>Octadecabacter antarcticus</i>	307	Sea ice and water. Antarctica, McMurdo Sound (77°53'N, 166°35'W)	Yes	Yes	
<i>Roseobacter</i> sp.	CCS2	Near Bodega Head park, California, USA	ND	Yes	
<i>Loktanella vestfoldensis</i>	SKA53	North Atlantic (2–5 m depth)	ND	Yes	
Unclassified	HTCC2083	Coast of Oregon, USA (10 m depth)	ND	Yes	
	HTCC2150	Coast of Oregon, USA (10 m depth)	ND	Yes	
<i>Oceanibulbus indolifex</i>	HEL-45	3 km off Helgoland Island. Germany, North Sea	ND	Yes	–
<i>Roseobacter denitrificans</i>	Och 114	Surface of macroalgae <i>Enteromorpha linza</i> . Australia	ND	Yes	–
<i>Roseobacter litoralis</i>	Och 149	Macroalgae (not identified)	ND	Yes	+
<i>Roseobacter</i> sp.	GAI-101	Coast of Georgia, USA	Yes	Yes	
<i>Sulfitobacter</i> sp.	NAS-14.1	Enriched for growth on dimethylsulfoniopropionate. North Atlantic (33°3'N, 74°3'W)	ND	Yes	
<i>Sulfitobacter</i> sp.	EE-36	Enriched for growth on lignocellulose. Coast of Georgia, USA	ND	Yes	
<i>Oceanicola batsensis</i>	HTCC2597	Bermuda Atlantic time series study station (10 m depth)	ND	Yes	–
<i>Oceanicola granulosus</i>	HTCC2516	Bermuda Atlantic time series study station (10 m depth)	ND	Yes ^c	

Abbreviations: CO, carbon monoxide; ND, not detected.

^aThe isolation details that are reported here relied on the detailed review by Brinkhoff *et al.* (2008).^b*Pelagibaca bermudensis* HTCC2601 is the only strain to have had RuBisCO genes detected (Newton *et al.*, 2010).^c*O. granulosus* HTCC2516 has a divergent *coxL* homologue (Figure 2), but no other *cox* genes were detected.

Figure 1 Phylogenetic analysis of the Marine Roseobacter Clade (MRC) strains compared in this study (Table 1). The tree is based on the almost complete 16S rRNA gene sequence alignments (> 1400 bp) using a neighbour-joining algorithm with a Maximum Composite Likelihood model and 1000 bootstrap replications. The circles on the nodes indicate bootstrap percentages; black circles indicate values $\geq 95\%$ and grey circles indicate $\geq 70\%$. The scale bar represents 0.02% sequence divergence. The 16S rRNA gene sequence from *Escherichia coli* DH1 (CP001637) was used as the outgroup. The stars indicate which MRC strain genomes had the form I and the form II *cox* gene clusters detected. *Roseovarius nubinihibens* ISM was the only MRC strain not to have any *cox* genes detected.

(Figure 2). The form I *coxL* genes and the putative form II *coxL* genes sequences are two distinct clusters, yet both groups are still separate from the closely related molybdenum hydroxylase nicotine dehydrogenase gene *ndhL*. The phylogeny of most of the MRC *coxL* gene sequences mimic the phylogeny of the MRC 16S rRNA genes, implying vertical inheritance of *coxL* genes (Figures 1 and 2). King (2003) made a wider PCR-based survey of *coxL* genes across strains from two phyla (*Proteobacteria* and *Actinobacteria*) and also showed monophyletic groupings similar to 16S rRNA gene phylogeny. The concordance of *coxL* gene and 16S rRNA gene phylogeny has significant practical implications if *coxL* genes are used as molecular markers instead of

16S rRNA genes to study CO oxidiser assemblage structure *in situ* (Dunfield and King, 2004, 2005; Cunliffe *et al.*, 2008).

There were fundamental differences in the organisation of the MRC strain *cox* genes between the two forms (Figure 3). CODH is encoded by three subunits, *coxL* along with *coxS*, which encodes an iron-sulfur protein and *coxM*, which encodes a flavin adenine dinucleotide-binding protein (Fuhrmann *et al.*, 2003). All the form I CODH encoding genes are in the order *coxMSL*, whereas all the form II CODH encoding genes are in the order *coxSLM*.

Associated non-CODH encoding *cox* genes showed marked differences in presence and order between the two forms and between MRC strains.

Figure 2 Phylogenetic analysis of the translated amino acid sequences of Marine Roseobacter Clade (MRC) *coxL* genes that encode the large subunit of carbon monoxide dehydrogenase. The tree is based on sequence alignments using a neighbour-joining algorithm with a Poisson correction model and 1000 bootstrap replications. The circles on the nodes indicate bootstrap percentages; black circles indicate values $\geq 95\%$ and grey circles indicate $\geq 70\%$. The scale bar represents 0.2% sequence divergence. The sequences from the non-MRC carboxydrotrophs *Oligotropha carboxidovorans* (X82447) and *Pseudomonas carboxydohydrogena* (AY463247) were included for comparison. The sequence from the non-carbon monoxide dehydrogenase molybdenum hydroxylase nictotine dehydrogenase (*ndhL*) (AF373840) was used as the outgroup.

Figure 3 Schematic diagram showing two examples of the organisation of *cox* genes from the Marine Roseobacter Clade genomes.

In all cases, the form I CODH genes were followed by *coxDEF*. Also present in some form I *cox* clusters were the genes *coxC* and *coxG*. The general consensus gene order was *coxCMSLDEFG* in the form I cluster. The obvious difference between the

forms I and II clusters is the loss of associated *cox* genes in the form II cluster (Figure 3). Some form II clusters contain the genes *coxE*, *coxF* and *coxG*; however, *coxD* and *coxC* were not detected. The associated form II *cox* genes were not always

Figure 4 Headspace CO concentrations of cultures of the Marine Roseobacter Clade strains compared in this study (Table 1). Error bars indicate s.d.'s.

flanking each other, as with the form I *cox* genes, and were instead punctuated with non-*cox* homologues. As a result, there is no consensus order of associated *cox* genes for the form II cluster.

O. granulosus HTCC 2516 has a divergent form II *coxL* homologue (discussed above) (Figure 2); however, no other CODH encoding *coxM*, *coxS* or associated *cox* genes were detected. Therefore, *O. granulosus* HTCC 2516 cannot be considered as a potential MRC CO oxidiser.

Little is known about the function of the associated *cox* genes and most research attention has been on *O. carboxidovorans*. Mutagenic disruption of *coxE* and *coxD* has implicated their role in the post-translational synthesis and maturation of CODH (Fuhrmann *et al.*, 2003; Pelzmann *et al.*, 2009). *coxG* mutants are able to synthesise a fully-functional CODH; however, CODH anchoring to the inner aspect of the cytoplasmic membrane (the normal location of the enzyme in *O. carboxidovorans*) was impaired (Fuhrmann *et al.*, 2003). Functional comparisons between *O. carboxidovorans* associated *cox* genes and MRC strain *cox* homologues, therefore, suggest that all the genes are present to synthesise a fully-functional CODH in the MRC form I *cox* gene clusters (Figure 3). The form II *cox* gene cluster, which is the ubiquitous form in MRC genomes (Table 1), cannot perform the post-translational modifications required to produce a functional CODH that is comparable to the form I CODH in *O. carboxidovorans*.

Only MRC strains that contain both the forms I and II *coxL* genes were able to oxidise CO (Table 1). During growth, CO uptake proceeded steadily down to the detection limit (approximately 20 p.p.m.) (Figure 4). All the MRC strains grew well in marine broth 2216 (Difco) with OD₆₀₀ >1 after 70 h. The headspace CO concentrations of the MRC strains

with only the form II *coxL* gene remained the same as those in control flasks (medium only). The ability to oxidise CO has only been previously confirmed in one MRC strain, *R. pomeroyi* DSS-3 (King, 2003; Moran *et al.*, 2004). Carbon monoxide oxidation has also been reported in another marine bacterioplankton group, the genus *Stappia*, which is related to the MRC. A previous study of several CO-oxidising marine *Stappia* spp. showed, using PCR retrieval of *coxL* genes, that both forms of the *coxL* gene were present in all of the strains analysed (Weber and King, 2007). Both *R. pomeroyi* DSS-3 (Moran *et al.*, 2004) and the *Stappia* spp. studied by Weber and King (2007) depleted headspace CO concentrations in the culture to sub-ambient concentrations. In this study, the initial CO concentration used was higher than the CO concentration found in seawater and was only followed to ~20 p.p.m. (the detection limit). Future experiments should aim to determine MRC strain activity under *in situ* conditions.

Carbon monoxide oxidation by CODH results in the production of carbon dioxide and reducing equivalents: $\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^-$ (King and Weber, 2007). Carboxydotrophic organisms, such as *O. carboxidovorans* and *P. carboxydohydrogena*, utilise both carbon dioxide and the energy to create biomass through the Calvin–Benson–Bassham cycle (Meyer and Schlegel, 1983; King and Weber, 2007). On the basis of genome annotation, most MRC strains lack any ribulose-1,5-bisphosphate carboxylase/oxygenase gene homologues. Only one MRC genome, *Pelagibaca bermudensis* HTCC2601, has had ribulose-1,5-bisphosphate carboxylase/oxygenase gene homologues detected (Table 1) (Newton *et al.*, 2010). The inability for MRC strains to assimilate the carbon dioxide from CO makes them carboxydovores, a type of chemolithoheterotroph, growing heterophically and using CO as a potential supplementary energy source.

The ecological implications of CO-supported chemolithoheterotrophy are important because populations that utilise this supplementary energy source will be able to use more organic carbon for biosynthesis, and therefore have probable fitness advantages over populations unable to access supplementary energy (Moran and Miller, 2007). Also, CO is produced in seawater by the photolysis of dissolved organic matter (Zuo and Jones, 1995) and is transferred into the atmosphere in which it is a secondary greenhouse gas (Daniel and Solomon, 1998). Fortunately, most of the CO that is produced in the oceans (up to 86%) is oxidised by microorganisms and is not able to escape into the atmosphere (Zafiriou *et al.*, 2003; Tolli and Taylor, 2005).

Conclusions

The distribution of the two CODH forms is uneven across the MRC strains included in this study; most strains only have the putative form II CODH.

There is genome-based evidence to suggest that the abundant form II CODH in the MRC lacks the associated *cox* genes required to synthesise an enzyme which is comparable to the definitive form I CODH in the archetypal CO oxidiser, *O. carboxidovorans*. This is supported empirically as from the nine MRC strains examined in this study only those strains that have both *coxL* gene forms could oxidise CO under the conditions tested. This study therefore raises doubt as to the validity of the assumption that CO oxidation is an almost universal feature of the MRC, as implied by recent reports (Moran *et al.*, 2007; Newton *et al.*, 2010).

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