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Molecular analysis of the distribution and phylogeny of dissimilatory adenosine-5′-phosphosulfate reductase-encoding genes (*aprBA*) among sulfur-oxidizing prokaryotes

Birte Meyer and Jan Kuever†

Max-Planck-Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

Correspondence
Jan Kuever
kuever@mpa-bremen.de

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Dissimilatory adenosine-5′-phosphosulfate (APS) reductase (AprBA) is a key enzyme of the dissimilatory sulfate-reduction pathway. Homologues have been found in photo- and chemotrophic sulfur-oxidizing prokaryotes (SOP), in which they are postulated to operate in the reverse direction, oxidizing sulfite to APS. Newly developed PCR assays allowed the amplification of 92–93 % (2.1–2.3 kb) of the APS reductase locus *aprBA*. PCR-based screening of 116 taxonomically divergent SOP reference strains revealed a distribution of *aprBA* restricted to photo- and chemotrophs with strict anaerobic or at least facultative anaerobic lifestyles, including *Chlorobiaceae*, *Chromatiaceae*, *Thiobacillus*, *Thiothrix* and invertebrate symbionts. In the AprBA-based tree, the SOP diverge into two distantly related phylogenetic lineages, Apr lineages I and II, with the proteins of lineage II (*Chlorobiaceae* and others) in closer affiliation to the enzymes of the sulfate-reducing prokaryotes (SRP). This clustering is discordant with the dissimilatory sulfite reductase (DsrAB) phylogeny and indicates putative lateral *aprBA* gene transfer from SRP to the respective SOB lineages. In support of lateral gene transfer (LGT), several beta- and gammaproteobacterial species harbour both *aprBA* homologues, the DsrAB-congruent ‘authentic’ and the SRP-related, LGT-derived gene loci, while some relatives possess exclusively the SRP-related *apr* genes as a possible result of resident gene displacement by the xenologue. The two-gene state might be an intermediate in the replacement of the resident essential gene. Collected genome data demonstrate the correlation between the AprBA tree topology and the composition/arrangement of the *apr* gene loci (occurrence of *qmoABC* or *aprM* genes) from SRP and SOP of lineages I and II. The putative functional role of the SRP-related APS reductases in photo- and chemotrophic SOP is discussed.

INTRODUCTION

Microbial sulfur oxidation is a key process in the oxidative half of the sulfur cycle in soil and water, and dominates as the major reaction, especially in extreme environments such as volcanic hot springs, solfataras and deep-sea hydrothermal vents. Reduced inorganic sulfur compounds such as sulfide, polysulfides, sulfur, sulfite, thiosulfate and various polythionates can serve as electron donors for the energy-generating systems in many photo- and chemotrophic species of the domain *Bacteria*, whereas this metabolic capability is restricted in the domain *Archaea* to representatives of the thermoacidophilic order *Sulfolobales* (Brune, 1995; Brüser *et al.*, 2000; Friedrich, 1998; Huber & Prangishvili, 2000; Nelson & Fisher, 1995; Overmann & Garcia-Pichel, 2001). The sulfur-oxidizing prokaryotes (SOP) are phylogenetically and physiologically diverse, differing in their abilities to utilize the various reduced sulfur compounds. As a consequence, the enzymic

†Present Address: Bremen Institute for Materials Testing, Paul-Feller-Strasse 1, D-28199 Bremen, Germany.

Abbreviations: APAT, adenylylsulfate : phosphate adenylyltransferase; APS, adenosine-5′-phosphosulfate; DGGE, denaturing gradient gel electrophoresis; DG-DGGE, double-gradient DGGE; LGT, lateral gene transfer; SAOR, sulfite : acceptor oxidoreductase; SDR, sulfur dioxygenase; SOB, sulfur-oxidizing bacteria; SOP, sulfur-oxidizing prokaryotes; SQR, sulfide : quinone oxidoreductase; SRP, sulfate-reducing prokaryotes.

The GenBank/EMBL/DDBJ accession numbers for the *aprBA* and 16S rRNA sequences of the species examined in this study are EF641902–EF641963 and EF675611–EF675615, respectively.

A supplementary table of the presence of genes encoding dissimilatory sulfite reductase and its functionally associated proteins in genome sequences of SOB, and two supplementary figures showing a phylogenetic consensus tree based on 16S rRNA gene sequences from the *apr*-containing SOB reference strains, and an AprB and AprA alignment showing indels among selected representatives of the major phylogenetic SOB lineages, are available with the online version of this paper.

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pathways used for dissimilatory sulfur oxidation have been found to be widely variable and may involve different intermediates (Brune, 1995; Brüser *et al.*, 2000; Friedrich, 1998; Friedrich *et al.*, 2001; Kletzin *et al.*, 2004; Nelson & Fisher, 1995). The oxidation of sulfide generally results in the formation of periplasmic and extracellular sulfur globules mediated by the activity of the ubiquitous enzyme sulfide : quinone oxidoreductase (SQR) (Griesbeck *et al.*, 2000; Theissen *et al.*, 2003). Its further oxidation to sulfate has been suggested to be mediated by the reverse-operating enzymes of the sulfate-reduction process, comprising the dissimilatory ATP sulfurylase, adenosine-5′-phosphosulfate (APS) reductase and sulfite reductase, which are ubiquitously present in the sulfate-reducing prokaryotes (SRP). Homologues have been shown to exist in several anoxygenic phototrophic and facultative anaerobic chemotrophic sulfur-oxidizing bacteria (SOB) (Dahl & Trüper, 1994; Hipp *et al.*, 1997; Taylor, 1994; Trüper & Fischer, 1982). Experimental evidence is accumulating that the reverse-acting sulfite reductase (DsrAB) and its functionally associated transmembrane redox complex DsrMKJOP are essential for the oxidation of sulfide or intermediately stored sulfur to sulfite (Dahl *et al.*, 2005; Sander *et al.*, 2006). Its subsequent oxidation to sulfate can be mediated by two different enzymes in SOB: (1) the APS reductase (Apr), catalysing the oxidative binding of sulfite to AMP, which generates APS as product; and (2) the sulfite : acceptor oxidoreductase (SAOR), catalysing the AMP-independent oxidation to sulfate without formation of intermediates (Brune, 1995; Brüser *et al.*, 2000; Kappler & Dahl, 2001). Sulfate is released from APS by the activity either of ATP sulfurylase (Sat) or adenylylsulfate : phosphate adenylyltransferase (APAT). Energy is yielded by substrate phosphorylation, with the former enzyme transferring the AMP moiety of APS onto pyrophosphate, which leads to ATP formation, while the latter enzyme uses phosphate for replacement and produces ADP (Brune, 1995; Brüser *et al.*, 2000).

Recent studies have confirmed that the dissimilatory APS reductases (irrespective of metabolic type) consist of two different subunits, which are proposed to form a 1 : 1 $\alpha\beta$-heterodimeric iron–sulfur flavoenzyme (AprBA). Only the α subunit, AprA, has structural similarity to the flavoprotein subunits of the succinate-dehydrogenase/fumarate-reductase family; however, this structural relationship is not reflected in sequence similarity (Fritz *et al.*, 2000, 2002; Schiffer *et al.*, 2006). The encoding genes, *aprBA*, have been cloned from sulfur-oxidizing *Allochromatium vinosum*; comparative sequence analysis confirmed their homology to the dissimilatory APS reductase of bacterial and archaeal SRP (Hipp *et al.*, 1997). However, the *in vivo* role of the reverse APS reductase, compared to that of the SAOR, in the dissimilatory oxidative sulfur metabolism of photo- and chemotrophic SOP (Dahl, 1996; Sanchez *et al.*, 2001) and its functional linkage to the photosynthetic and respiratory electron transport chain are still unresolved. For SRP, there is increasing evidence that the Qmo redox complex, consisting of one membrane-integral (QmoC) and two cytoplasmic (QmoAB) proteins, acts as a menaquinol/APS reductase oxidoreductase (Haveman *et al.*, 2004; Pires *et al.*, 2003).

Previous phylogenetic analyses of enzymes of the sulfate-reduction pathway (Apr and Dsr) have been restricted to SRP, and have revealed that multiple lateral gene transfer (LGT) events have affected their evolutionary path (Friedrich, 2002; Klein *et al.*, 2001) (Meyer & Kuever, 2007). The aims of this study were (1) the comprehensive molecular investigation of *aprBA* distribution among SOP by examining 116 representatives of photo- and chemotrophic sulfur-oxidizing *Archaea* and *Bacteria*, and (2) the phylogenetic analysis of the AprBA sequences to reveal potential LGTs affecting the SOP and to elucidate the origin and evolution of the sulfite-oxidation process. Accordingly, novel PCR assays were developed that enabled the amplification and direct sequencing of 92–93 % (equivalent to 2.1–2.3 kb) of the reverse APS reductase gene region to establish a new *aprBA* database from the major taxonomic lineages of SOP. The results of this work also provide a framework for future molecular ecological studies to investigate the microbial community of the sulfur cycle by functional gene analysis.

METHODS

Micro-organisms. The investigated reference strains of photo- and chemotrophic SOB (listed in Table 1) were obtained from the DSMZ (Braunschweig, Germany). Genomic DNA of *Chlorobiaceae* and several *Chromatiaceae* were received from the culture collection of J. Imhoff (University of Kiel) Extracted genomic DNAs of invertebrate tissues were provided by N. Dubilier, Max-Planck-Institute for Marine Microbiology, (*Inanidrilus* spp., *Bathymodiolus azoricus* and *Bathymodiolus brevior*), A. D. Nussbauer, University of Vienna, (*Riftia pachyptila*, *Bathymodiolus thermophilus*, *Calyptogena magnifica* and *Oasisia* sp.) and C. Borowski, Max-Planck-Institute for Marine Microbiology, (*Ifremeria nautilei*). Harvested cells of *Beggiatoa* spp., *Aquaspirillum* spp., *Macromonas bipunctata* strain D-408 and *Spirochaeta* spp. were received from G. Dubinina, Winogradsky Institute of Microbiology. The SOB strain 'manganese crust' was isolated from enrichment cultures of sediment and seawater samples from the Caribbean Sea (Cariblux project, SO-154).

DNA isolation. Genomic DNA from the investigated reference strains was obtained by applying the DNAeasy kit (Qiagen) or the NUCLEOBOND kit (Macherey–Nagel) according to the manufacturers' instructions. The DNA concentration and quality were estimated spectrophotometrically, while its integrity was examined visually by gel electrophoresis on 0.8 % (w/v) agarose gels run in 1 × Tris/borate/EDTA (TBE) buffer and followed by ethidium bromide staining (0.5 μg ml^{-1}).

PCR amplification of *aprBA* and 16S rRNA genes. The forward primers AprB-1-FW and AprB-3-FW (with different target sites in the *aprB* gene) were used in combination with the reverse primer AprA-5-RV for amplification of a 1.2–1.3 kb and a 1.2–1.1 kb *aprBA* gene fragment, respectively. The forward primer AprA-1-FW was combined with the reverse primers AprA-9-RV or AprA-10-RV (identical target site in the *aprA* gene), which yielded a 1.4 kb *aprA* amplicon from the 5′-terminal region of *aprA*. The *aprBA* and *aprA* amplicons

Table 1. PCR amplification results for *aprBA* gene fragments from genomic DNA of sulfur-oxidizing reference strains

Species*	Strain†	PCR product obtained with primer set‡			Phylogenetic affiliation and length of the *aprBA* sequence§		GenBank accession no. *aprBA*
		AprB-1-FW/ AprA-5-RV	AprB-3-FW/ AprA-5-RV	AprA-1-FW/ AprA-9-RV or AprA-10-RV	Apr lineage I	Apr lineage II	
Archaea							
Phylum *Crenarchaeota*, *Thermoprotei*							
Sulfolobaceae							
Acidianus ambivalens	3772^T	− (52)	− (54)	− (54)	−	−	−
Metallosphaera sedulae	5348^T	− (52)	− (54)	− (54)	−	−	−
Metallosphaera prunae	10039^T	− (52)	− (54)	− (54)	−	−	−
Sulfolobus metallicus	6482^T	− (52)	− (54)	− (54)	−	−	−
Bacteria							
Phylum *Chloroflexi*, *Chloroflexi*							
Chloroflexaceae							
Chloroflexus aggregans	9485	− (54)	− (54)	− (54)	−	−	−
Phylum *Chlorobi*, *Chlorobia*							
Chlorobiaceae							
1 *Prosthecochloris aestuarii***	271^T	− (54)	− (54)	− (54)	−	−	−
Prosthecochloris sp.**	*2K*	− (54)	− (54)	− (54)	−	−	−
*Prosthecochloris vibrioforme***	260	− (54)	− (54)	− (54)	−	−	−
Prosthecochloris vibrioforme **	1678	− (54)	− (54)	− (54)	−	−	−
2a *Chlorobium luteolum***	273^T	− (54)	− (54)	− (54)	−	−	−
*Chlorobium luteolum***	262	− (54)	− (54)	− (54)	−	−	−
2b *Chlorobium phaeovibrioides***	269^T	− (54)	− (54)	− (54)	−	−	−
*Chlorobium phaeovibrioides***	265	− (54)	− (54)	− (54)	−	−	−
*Chlorobium phaeovibrioides***	261	− (54)	− (54)	− (54)	−	−	−
*Chlorobium phaeovibrioides*** ‖	270	− (54)	− (54)	− (54)	−	−	−
3a *Chlorobium phaeobacteroides***	266^T	− (54)	− (54)	− (54)	−	−	−
*Chlorobium clathratiforme***	5477^T	+ (57)	+ (63)	+ (63)	−	II: 2182	EF641906
'*Chlorobium ferrooxidans*'	13031^T	− (54)	ND	− (54)	−	−	−
3b *Chlorobium limicola*** ‖	245^T	− (54)	− (54)	− (54)	−	−	−
*Chlorobium limicola***	246	− (54)	− (54)	− (54)	−	−	−
*Chlorobium limicola***	*2323*	− (54)	− (54)	− (54)	−	−	−
*Chlorobium limicola***	1855	+ (63)	+ (63)	+ (63)	−	II: 2166	EF641905
*Chlorobium limicola*** ‖	257	+ (63)	+ (63)	+ (63)	−	II: 2177	EF641903
*Chlorobium limicola***	247	− (54)	− (54)	− (54)	−	−	−
*Chlorobium limicola***	248	− (54)	− (54)	− (54)	−	−	−
4a *Chlorobaculum parvum***	263^T	− (54)	− (54)	− (54)	−	−	−
*Chlorobaculum parvum*** ‖	*2352*	− (54)	− (54)	− (54)	−	−	−
4b *Chlorobaculum limnaeum***	1677	+ (63)	+ (63)	+ (63)	−	II: 2168	EF641904
*Chlorobaculum thiosulfatiphilum*** ‖	249^T	± (63)	ND	± (63)	−	II: 396	EF669482
*Chlorobaculum thiosulfatiphilum***	*2322*	+ (63)	+ (63)	+ (63)	−	II: 2226	EF641902
Phylum *Proteobacteria*, *Alphaproteobacteria*							
Rhodospirillaceae							
Rhodospirillum photometricum	122^T	− (54)	ND	− (54)	−	−	−
Rhodobacteraceae							
Rhodothalassium salexigens	2132^T	− (54)	ND	− (54)	−	−	−
Rhodovulum adriaticum	2781	− (54)	ND	− (54)	−	−	−
Rhodovulum sulfidophilum	1374^T	− (54)	ND	− (54)	−	−	−
Bradyrhizobiaceae							
Rhodoblastus acidophilus	137^T	− (54)	ND	− (54)	−	−	−
Hyphomicrobiaceae							
Blastochloris viridis	133^T	− (54)	ND	− (54)	−	−	−

Table 1. cont.

Species*	Strain†	PCR product obtained with primer set‡			Phylogenetic affiliation and length of the *aprBA* sequence§		GenBank accession no. *aprBA*
		AprB-1-FW/AprA-5-RV	AprB-3-FW/AprA-5-RV	AprA-1-FW/AprA-9-RV or AprA-10-RV	Apr lineage I	Apr lineage II	
Rhodobiaceae							
Rhodobium marinum	2698^{T}	− (54)	ND	− (54)	−	−	−
Phylum *Proteobacteria*, *Betaproteobacteria*							
Hydrogenophilaceae							
Thiobacillus aquaesulis	4255^{T}	+ (55)	+ (63)	± (63)	−	II: 2314	EF641916
Thiobacillus denitrificans	12475^{T}	+ (55)	+ (63)	± (63)	I: 1197	II: 2262	EF641952, EF641924
Thiobacillus denitrificans	739	± (63)	+ (63)	± (63)	I: 553	II: 2218	EF641955, EF641923
Thiobacillus denitrificans¶	807	± (63)	ND	± (63)	−	II: 2215	EF641922
Thiobacillus plumbophilus	6690^{T}	± (55)	± (63)	± (63)	I: 1183	II: 2317	EF641956, EF641917
Thiobacillus thioparus¶	505^{T}	± (55)	+ (63)	+ (63)	I: 1152	II: 2275	EF641954, EF641920
Neisseriaceae							
Aquaspirillum sp. strain D-412	−	− (55)	ND	− (55)	−	−	−
Aquaspirillum sp. strain D-415	−	− (55)	ND	− (55)	−	−	−
Phylum *Proteobacteria*, *Gammaproteobacteria*							
Chromatiaceae							
Allochromatium minutissimum‖	1376^{T}	± (63)	+ (63)	± (63)	I: 2090	−	EF641963
Allochromatium vinosum‖	180^{T}	+ (63)	+ (63)	+ (63)	I: 2168	−	−
Allochromatium warmingii‖	173^{T}	± (55)	+ (63)	± (63)	I: 2140	−	EF641931
*Chromatium okenii***	*6010*	± (63)	+ (63)	± (63)	I: 2122	−	EF641935
Halochromatium glycolicum	11080^{T}	± (63)	± (63)	+ (63)	I: 2142	−	EF641934
Halochromatium salexigens	4395^{T}	+ (63)	± (63)	+ (63)	I: 2127	−	EF641933
Isochromatium buderi	176^{T}	− (55)	ND	− (54)	−	−	−
*Isochromatium buderi***	*5612*	− (55)	ND	− (54)	−	−	−
*Lamprocystis purpurea***	4197^{T}	+ (63)	+ (63)	− (55)	−	II: 1285	EF641909
*Lamprocystis roseopersicina***	*4510*	+ (63)	ND	+ (63)	I: 2159	−	EF641939
Marichromatium gracile	203^{T}	− (55)	ND	− (54)	−	−	−
Marichromatium purpuratum	1591^{T}	− (55)	ND	− (54)	−	−	−
Rhabdochomatium marinum	5261^{T}	+ (63)	+ (63)	± (63)	I: 2159	−	EF641947
Thermochromatium tepidum	3771^{T}	± (63)	+ (63)	± (63)	I: 2121	−	EF641936
Thiocapsa pendens	236^{T}	+ (55)	ND	− (55)	−	II: 1298	EF641914
*Thiocapsa rosea***	235^{T}	− (57)	− (63)	− (55)	I: 371	−	EF641961
Thiocapsa roseopersicina	217^{T}	+ (63)	+ (63)	+ (63)	I: 1197	II: 2285	EF641937, EF641907
*Thiocapsa roseopersicina***	*4210*	+ (63)	+ (63)	+ (63)	I: 1197	II: 2298	EF641938, EF641908
*Thiococcus pfennigii***	226^{T}	+ (63)	ND	± (63)	I: 2129	−	EF641942
Thiococcus pfennigii	227	+ (63)	+ (63)	+ (63)	I: 2138	−	EF641943
Thiococcus pfennigii	228	± (63)	+ (63)	± (63)	I: 2126	−	EF641944
Thiocystis gelatinosa	215^{T}	+ (63)	+ (55)	+ (63)	−	II: 2327	EF641911
Thiocystis violacea‖	207^{T}	+ (63)	+ (63)	+ (63)	I: 1197	II: 2238	EF641948, EF641912
Thiocystis violacea	214	+ (63)	ND	+ (63)	I: 2128	−	EF641949
Thiocystis violascens	198^{T}	+ (63)	+ (63)	+ (55)	−	II: 2251	EF641910
*Thiodictyon bacillosum***	234^{T}	+ (63)	ND	+ (55)	−	II: 2155	EF641915
Thiodictyon sp. strain F4	−	± (50)	ND	± (50)	−	II: 2321	EF641921
Thiohalocapsa halophila	6210^{T}	+ (63)	+ (63)	+ (63)	I: 2172	−	EF641932

Table 1. cont.

Species*	Strain†	PCR product obtained with primer set‡			Phylogenetic affiliation and length of the *aprBA* sequence§		GenBank accession no. *aprBA*
		AprB-1-FW/ AprA-5-RV	AprB-3-FW/ AprA-5-RV	AprA-1-FW/ AprA-9-RV or AprA-10-RV	Apr lineage I	Apr lineage II	
Thiolamprovum pedioforme	3802^T	+ (60)	+ (63)	+ (63)	I: 2163	−	EF641941
Thiorhodococcus minor	11518^T	+ (63)	+ (63)	± (55)	I: 1205	II: 1250	EF641950, EF641913
Thiorhodovibrio winogradskyi	6702^T	+ (63)	ND	+ (63)	I: 2094	−	EF641946
Ectothiorhodospiraceae							
Ectothiorhodospira mobilis†	4180	− (54)	ND	− (54)	−	−	−
Ectothiorhodospira shaposhnikovii	243^T	− (54)	ND	− (54)	−	−	−
Halothiobacillaceae							
Halothiobacillus kellyi	13162^T	− (50)	− (50)	− (50)	−	−	−
Halothiobacillus neapolitanus	581^T	− (50)	− (50)	− (50)	−	−	−
Thiovirga sulfuroxydans sp. strain A7	−	− (58)	ND	ND	−	−	−
Thiotrichaceae							
Beggiatoa alba	1416^T	− (54)	ND	− (54)	−	−	−
Beggiatoa leptomitiformis strain D-401	−	− (54)	ND	− (54)	−	−	−
Beggiatoa leptomitiformis strain D-402	−	− (54)	ND	− (54)	−	−	−
Leucothrix mucor	2157^T	− (55)	− (50)	− (50)	−	−	−
Leucothrix mucor	621	− (55)	− (50)	− (50)	−	−	−
Macromonas bipunctata strain D-408	−	− (55)	ND	− (55)	−	−	−
Thiothrix nivea	5205^T	+ (54)	− (63)	+ (56)	−	II: 2277	EF641919
Thiothrix sp.	12730	+ (54)	− (63)	+ (56)	−	II: 2318	EF641918
Piscirickettsiaceae							
Thiomicrospira frisia	12351^T	− (54)	ND	− (54)	−	−	−
Thiomicrospira kuenenii	12350^T	− (54)	ND	− (54)	−	−	−
Thiomicrospira sp.	13163	− (54)	ND	− (54)	−	−	−
Thiomicrospira sp.	13164	− (54)	ND	− (54)	−	−	−
Thiomicrospira sp.	13189	− (54)	ND	− (54)	−	−	−
Thiomicrospira sp.	13190	− (54)	ND	− (54)	−	−	−
Uncertain affiliation							
'*Thiobacillus prosperus*'	5130^T	− (50)	− (50)	− (50)	−	−	−
Invertebrate symbionts and free-living relatives							
Bathymodiolus azoricus symbiont	−	± (54)	ND	± (54)	I: 2142	−	EF641959
Bathymodiolus brevior symbiont	−	± (54)	ND	± (54)	I: 2163	−	EF641958
Bathymodiolus thermophilus symbiont	−	± (54)	ND	± (54)	I: 2169	−	EF641960
Calyptogena magnifica symbiont	−	± (55)	ND	± (55)	I: 982	−	EF669484
Ifremeria nautilei symbiont	−	+ (55)	+ (55)	± (55)	−	II: 1304	EF641929
Inanidrilus exumae symbiont	−	+ (57)	ND	± (52)	I: 1198	II: 1331	EF641957, EF641927
Inanidrilus leukodermatus symbiont	−	+ (57)	ND	± (52)	−	II: 2225	EF641926
Inanidrilus makropetalos symbiont	−	+ (57)	ND	± (52)	−	II: 2249	EF641925
Oasisia sp. symbiont	−	± (54)	ND	± (52)	I: 396	−	EF641962
Riftia pachyptila symbiont	−	+ (57)	ND	± (55)	−	II: 2210	EF641928
Sulfur-oxidizing bacterium OAII2#	−	± (54)	ND	± (53)	I: 2105	−	EF641953
Sulfur-oxidizing bacterium OBII5	−	± (54)	ND	± (53)	−	−	−
Sulfur-oxidizing bacterium ODIII5	−	+ (55)	ND	± (55)	I: 2161	−	EF641951
Sulfur-oxidizing bacterium ODI4	−	− (55)	ND	− (54)	−	−	−
Sulfur-oxidizing bacterium NDII1.2		− (55)	ND	− (54)	−	−	−
Sulfur-oxidizing bacterium 'manganese crust'	−	± (50)	ND	± (50)	I: 1288	−	EF641930

Table 1. cont.

Species*	Strain†	PCR product obtained with primer set‡			Phylogenetic affiliation and length of the *aprBA* sequence§		GenBank accession no. *aprBA*
		AprB-1-FW/ AprA-5-RV	AprB-3-FW/ AprA-5-RV	AprA-1-FW/ AprA-9-RV or AprA-10-RV	Apr lineage I	Apr lineage II	
Phylum *Proteobacteria*, *Epsilonproteobacteria*							
Helicobacteraceae							
Sulfurimonas denitrificans	1251^T	− (54)	ND	− (54)	−	−	−
Phylum *Spirochaeta*, *Spirochaetes*							
Spirochaetaceae							
Spirochaeta sp. strain P	−	− (55)	ND	− (55)	−	−	−
Spirochaeta sp. strain BM	−	− (55)	ND	− (55)	−	−	−
Spirochaeta sp. strain M-6	−	− (55)	ND	− (55)	−	−	−

*Taxonomic classification of investigated SRP species according to the Taxonomic outline of the prokaryotes, Bergey's Manual of Systematic Bacteriology, 2nd edition, release 5.0, May 2004 (http://dx.doi.org/10.1007/bergeysoutline); genomic DNA of sulfur-oxidizing reference strains marked with ** were received from J. Imhoff's laboratory (Kiel, Germany).
†DSMZ strain numbers of investigated species (Imhoff laboratory internal numbers in italic type); −, not deposited in a culture collection; T, type strain.
‡PCR amplification results with the respective primer pair: +, correct-sized amplicon without by-products; ±, correct-sized amplicon with by-products; −, no amplicon obtained; ND, PCR amplification not determined. The initial PCR annealing temperature (°C) is given in parentheses.
§Length of assembled *aprBA* gene fragment PCR product and phylogenetic assignment of the deduced AprBA sequence to the Apr lineages I and II.
||Proposed presence of dissimilatory APS reductase in respective species based on enzyme activity assay (see Dahl & Trüper, 1994, and references therein).
¶Proposed presence of dissimilatory APS reductase in respective species based on enzyme activity assay (see Taylor, 1994, and references therein).
#Proposed presence of dissimilatory APS reductase in respective species based on enzyme activity assay (see Kuever *et al.*, 2002).
††Uncertain taxonomic classification (synonym *Ectothiorhodospira marismortui*).

overlap in sequence by ~390 bp (corresponding to nucleotide positions 3471–3859 in the *sat–aprMBA* operon of *Allochromatium vinosum*; Table 2).

PCR assays were performed with the RED*Taq* DNA polymerase kit (Sigma–Aldrich). Reaction mixtures (50 µl total volume) contained 5 µl 10 × RED*Taq* PCR reaction buffer (with 11 mM $MgCl_2$), 5 µl 10 × BSA solution (3 mg ml^{-1}), 200 µM deoxynucleoside triphosphates (dNTPs) mixture, 1 µM of each primer and 10–100 ng genomic DNA as template (water was used as a negative control in all PCR amplifications). PCR amplification was performed in a thermocycler (Eppendorf) with an initial denaturation step of 95 °C for 5 min followed by the addition of 2.5 U RED*Taq* DNA polymerase to each PCR assay (hot start PCR). Subsequently, a 'touchdown' thermal profile was performed (35 cycles in total): 95 °C for 60 s (denaturation), 63 °C for 90 s ('touchdown': the annealing temperature was lowered at every cycle of the first 20 cycles by 0.5 °C to a final temperature of 53 °C, after which 15 additional cycles were carried out) and 72 °C for 120 s (elongation). Amplification was completed by a final elongation step at 72 °C for 10 min. The initial annealing temperature of the 'touchdown' was altered in a range between 63 and 55 °C to optimize the amplification results.

16S rRNA gene fragments were amplified using the primer sets GM3F/GM4R and GM5F-GC clamp/907R [for subsequent denaturing gradient gel electrophoresis (DGGE) analysis] with the PCR conditions described elsewhere (Muyzer *et al.*, 1995).

Double-gradient (DG) DGGE analysis of PCR-amplified 16S rRNA gene fragments. DG-DGGE was applied to improve the quality of 16S rDNA band separation and resolution. An acrylamide gradient from 6 to 8 % acrylamide/bisacrylamide stock solution, 37.5 : 1 (v/v) (Bio-Rad), was superimposed over a collinear denaturant gradient from 20 to 70 % of denaturant [100 % denaturant corresponds to 7 M urea and 40 % formamide (v/ v), deionized with AG501-X8 mixed bed resin (Bio-Rad)]. Gradients were formed using a Bio-Rad Gradient Former model 385. PCR samples were applied to the gels in aliquots of 20 µl per lane. Further analysis was performed as described elsewhere (Muyzer *et al.*, 1995) using the D-CODE and D-GENE systems (Bio-Rad) for electrophoresis runs in 1 × Tris/acetate/EDTA (TAE) buffer at 60 °C for 3.5 h at 200 V. After staining with ethidium bromide (0.5 µg ml^{-1}), DNA bands were visualized on a UV transillumination table (Biometra), excised from the polyacrylamide gel, eluted in 50 µl Tris/HCl, pH 8.0, and reamplified using the original PCR conditions and primer pair without a GC clamp.

Cloning of PCR products. The *aprBA* and 16S rRNA amplicons were ligated into pCR2.1-TOPO vectors (TOPO TA cloning systems; Invitrogen) and transformed into chemically competent *Escherichia coli* TOP10 cells as recommended by the manufacturer. Clone plasmids were screened for correct-sized inserts by PCR amplification followed by RFLP analysis of the amplicons. Cloned plasmids with different digestion patterns were selected for sequencing and recovered with the QIAprep spin kit (Qiagen).

Nucleotide sequencing. The *aprBA*, *aprA* and 16S rDNA PCR products of the expected sizes were purified using QIAquick PCR purification, the QIAquick gel extraction kit (Qiagen) or the

Table 2. PCR primers utilized for amplification of the *aprBA* and *aprA* gene fragments

Primer	Sequence (5′→3′)*	Primer binding site†	Reference
AprB-1-FW	TGC GTG TA**Y** AT**H** TG**Y** CC	2653–2669	‡
AprB-3-FW	AC**M** **W**GT GCT GGG AGT GCT AC	2726–2745	This study
AprA-1-FW	TGG CAG ATC ATG AT**Y** MA**Y** GG	3471–3490	‡
AprA-5-RV	GCG CCA AC**Y** GG**R** CC**R** TA	3843–3859	‡
AprA-9-RV	C**K**G **W**AG TAG TA**R** CC**S** GG**S** YA	4811–4831	This study
AprA-10-RV	C**K**G **W**AG TAG **W**A**R** CC**R** GG**R** TA	4811–4831	‡

*Degenerate positions are in bold type.

†Corresponding nucleotide positions of the *sat–aprMBA* operon of *Allochromatium vinosum* (GenBank accession no. U84759).

‡Meyer & Kuever (2007).

Perfectprep gel cleanup sample kit (Eppendorf,), following the suppliers' recommendations. All PCR products were sequenced directly in both directions using the respective amplification primers and the ABI BigDye terminator cycle sequencing kit (Applied Biosystems). Sequencing reactions were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Sequence analysis tools and phylogenetic tree inference. The nucleotide sequence data for the *aprBA* and *aprA* amplicons were assembled (2.1–2.3 kb total length) and manually corrected using the BioEdit (version 7.0.5) sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). BLAST searches for homologues (AprBA and DsrAB) were performed at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Searches on the preliminary sequence data of ongoing SOB and SRP genome sequencing projects were performed at The Institute for Genomic Research website (http://www.tigr.org) and at the US Department of Energy (DOE) Joint Genome Institute website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). The AprBA and DsrAB sequences were aligned by using the web server Tcoffee@igs (http://igs-server.cnrs-mrs.fr/Tcoffee/). The AprBA and DsrAB datasets were phylogenetically analysed with the tree inference methods included in the ARB software package (http://www.arb-home.de). Alignment regions of insertions and deletions (indels) were not considered. Unrooted phylogenetic trees were calculated based on two AprBA datasets of 379 and 701, and a DsrAB dataset of 740 compared amino acid positions, using the ARB implemented program package (distance matrix, Fitch analysis; maximum-parsimony, ProtPars; maximum-likelihood, ProML) and the PhyML program (maximum-likelihood method; http://atgc.lirmm.fr/phyml). Maximum-likelihood trees were constructed using the Whelan and Goldman (WAG) or Jones, Taylor and Thornton (JTT) amino acid substitution model matrices. The robustness of inferred trees was tested by bootstrap analysis with 100 resamplings using the PhyML program.

Prediction of potential promoters, termination sites and gene arrangement in operons was performed using the web versions FGENESB, BPROM and BTERM of the Softberry program package (http://www.softberry.com/berry.phtml). Secondary structure analysis and transmembrane helix prediction were done using programs available at http://us.expasy.org/tools/#secondary.

Southern blot analysis. Identical amounts of genomic DNA (5 μg) from sulfur-oxidizing and sulfate-reducing bacteria (Table 3) were digested at 37 °C with *Hin*dIII and *Eco*RI overnight, precipitated by ethanol, electrophoresed on 0.8 % 1 × TAE buffer at 100 V for 3 h, transferred to positively charged nylon membranes (Hybond-N+ filter, Amersham) by capillary neutral transfer and immobilized by UV cross-linking (Transilluminator; Biometra). The *aprA* gene DNA probes (0.4 kb in length) were radioactively labelled with [α-^{32}P]dCTP by the random-priming method using the HexaLabel DNA labelling kit (MBI Fermentas) according to the manufacturer's instructions. The membranes were placed into glass hybridization bottles and prehybridized in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 8.0), 50 % formamide, 0.1 % sarcosyl, 7 % SDS, 50 mM phosphate buffer, pH 7.0, and 2 % casein ('Church' hybridization solution) at 50 °C for 1 h in a hybridization oven (Biometra). Subsequently, a freshly denaturated, labelled DNA probe was added to the prehybridization solution followed by incubation for 12–16 h at 50 °C under slow-speed rotation. The membranes were washed twice at 50 °C for 30 min in 0.1 × SSC/0.1 % SDS, exposed to PhosphorImaging screen cassettes (Molecular Dynamics), scanned with a Typhoon Variable Mode Imager and processed with ImageQuant software (Amersham). The membranes were stripped by two incubations for 15 min in probe-stripping solution (consisting of 0.4 M NaOH and 0.1 % SDS) at 37 °C under permanent agitation and reprobed, starting from the prehybridization step of the hybridization procedure.

RESULTS

Amplification of *aprBA* genes by PCR from SOB

In this study, 116 archaeal or bacterial, photo- or chemolithotrophic reference strains were investigated for the presence of *aprBA* genes. Previous studies have reported the association of invertebrates with a single thiotrophic symbiont phylotype (Blazejak *et al.*, 2006; Bright & Giere, 2005; Cavanaugh *et al.*, 2001; Nelson & Fisher, 1995), which is supported by the single Apr sequences obtained from the investigated species (except *Inanidrilus exumae*). The results of the PCR-based survey are summarized in Table 1 and demonstrate the high species diversity coverage of the primers. In general, the four different primer sets yielded correct-sized PCR products when higher annealing temperatures (>55 °C) were applied in the assays. However, in some cases (e.g. invertebrate symbionts) the annealing temperature had to be lowered (from 55 to 50 °C) to obtain a sufficient amount of *apr* amplicons for direct sequencing. The comparison of the primer sequences to newly available genomic *aprBA* sequences (e.g. *Candidatus* Ruthia magnifica

Table 3. Determination of *apr* gene locus number in SOB

Cloning assay results for the *aprBA* amplicons from SOB species for which direct sequencing failed are shown; the potential presence of contaminants in the investigated SOB cultures was checked by cloning and DGGE analysis of PCR-amplified 16S rRNA gene fragments.

Species	DSM*	Gene locus number according to sequence analysis of cloned *aprBA* amplicons†	DGGE analysis and cloning assay results of 16S rRNA gene amplicons‡	
		AprB-1-FW or AprB-3-FW/AprA-5-RV§	GM5F-GC clamp/907R§	GM3F/GM4R§
Gammaproteobacteria				
Thiocapsa roseopersicina	217	2 (Apr lineages I and II)	Four DGGE bands‖	ND
Thiocapsa roseopersicina	*4210***	2 (Apr lineages I and II)	Two DGGE bands‖	ND
Thiocystis violacea	207	2 (Apr lineages I and II)	Two DGGE bands: seq. nearly identical to reference strain	Two seq. nearly identical to reference strain
Thiorhodococcus minor	11518	2 (Apr lineages I and II)	Two DGGE bands: seq. nearly identical to reference strain	Two seq. nearly identical to reference strain
Invertebrate symbionts				
Inanidrilus exumae	–	2 (Apr lineages I and II)	Two DGGE bands‖	ND
Betaproteobacteria				
Thiobacillus denitrificans	12475	2 (Apr lineages I and II)	One DGGE band: seq. identical to reference strain	One seq. identical to reference strain
Thiobacillus denitrificans	739	2 (Apr lineages I and II)	One DGGE band: seq. identical to reference strain	ND
Thiobacillus plumbophilus	6690	2 (Apr lineages I and II)	One DGGE band: seq. identical to reference strain	One seq. identical to reference strain
Thiobacillus thioparus	505	2 (Apr lineages I and II)	One DGGE band: seq. identical to reference strain	ND

*Cultures obtained from culture collection of J. Imhoff (laboratory internal numbers in italic type) are marked with **; –, not deposited in a culture collection.
†Phylogenetic assignment of AprBA sequences to Apr lineages I and II in parentheses.
‡Abbreviations: ND, 16S rDNA amplicon cloning assays of respective SOB species were not performed; seq., sequence(s).
§Primer sets used to generate amplicons. For primer sets GM5F/907R and GM3F/GM4R, see Muyzer *et al.* (1995).
‖No pure culture of reference strains according to 16S rDNA-based DGGE analysis: besides the sulfur-oxidizing reference strain, additional 16S rDNA sequence(s) indicated the presence of non-sulfur-oxidizing species (BLAST search results) in the investigated cultures.

and *Olavius algarvensis* symbionts) (Newton *et al.*, 2007; Woyke *et al.*, 2006) revealed the presence of single mismatches to the strictly conserved target sites. However, as reported for 16S rRNA-based studies (Kwok *et al.*, 1990; Simsek & Adnan, 2000), the presence of single mismatches at internal or 5′-end sequence positions in the novel PCR primers did not affect their PCR efficiency. Thus, the absence of *apr* amplicons most likely reflects the absence of the APS reductase genes in the respective SOB species. Overall, the occurrence of *aprBA* genes in SOB seemed to be restricted to most *Chromatiaceae* species, a few representatives of the *Chlorobiaceae*, *Thiobacillus* spp. (*Betaproteobacteria*), *Thiothrix* spp. and the sulfur-oxidizing symbionts, including their free-living relatives (*Gammaproteobacteria*).

Number of *apr* gene loci in SOB

Interestingly, direct nucleotide sequencing of *aprBA* and *aprA* amplicons of some SOB species (Table 3) failed due to high levels of sequence ambiguities. Investigation of the genome of *Thiobacillus denitrificans* ATCC 25259 (Beller *et al.*, 2006) revealed the existence of two *apr* gene loci of differing sequence. Therefore, the previous SOB species were checked for the presence of multiple *apr* genes by cloning assays of the *aprBA* PCR products and subsequent RFLP analysis of the recombinant plasmids. The assays revealed the existence of two *aprBA* genes of differing sequence in genomic DNA from *Thiocapsa roseopersicina* (DSM 217 and *4210*), *Thiocystis violacea* and *Thiorhodococcus minor*, *Thiobacillus denitrificans* (DSM 12475 and 739), *Thiobacillus thioparus* and *Thiobacillus plumbophilus* cultures, and the Gamma-4 symbiont of *Inanidrilus exumae* (see Table 3). The GM3F/GM4R-amplified 16S rRNA gene fragments of these species were analysed by cloning assays (followed by RFLP analysis), while all investigated SOB cultures were checked by 16S rDNA-based DGGE analysis; the results confirmed the absence of putative sulfur-oxidizing, *apr*-harbouring contaminants in the examined reference strain cultures of this study.

Unexpectedly, PCR amplification of *apr* gene fragments was unsuccessful for most studied *Chlorobiaceae* (see Table 1). Therefore, Southern blot experiments were performed with *Chlorobium* spp. [subclusters 2a and 3b (Imhoff, 2003)] by using different, radioactively labelled *aprA* probes (Table 4; *aprA* probe sequence was verified by direct sequencing of an identical non-labelled PCR product). The Southern blot analyses confirmed the results of the PCR assays for the *Chlorobiaceae*. In addition, the appearance of two distinct hybridization signals with the *Thiocapsa roseopersicina*-specific probe supported the existence of two *aprA* genes in *Thiocapsa roseopersicina*, as suggested by the PCR and cloning assays (Table 1).

Phylogeny of dissimilatory APS reductase of SOB

The 63 partial (Table 1) and 27 full-length AprBA sequences of sulfur-oxidizing reference strains (from public databases; Table 5) were integrated into the pre-existing AprBA database of SRP species (Meyer &, 2007). Phylogenetic trees were constructed based on (1) 379 compared positions (sequence data of the *aprBA* amplicon) (Fig. 1) and (2) 701 compared positions (assembled sequence data of the *aprBA* and *aprA* amplicons) (Fig. 2). In contrast to the DsrAB phylogeny, which clearly demonstrates the evolutionary divergence of the proteins from sulfate/sulfite reducers and sulfur oxidizers into two separate clusters (Fig. 3), the AprBA sequences of SOB separated into two distinct lineages, Apr lineages I and II. While the oxidative-operating AprBA proteins of lineage I formed a distinct, DsrAB-congruent cluster of SOB (less than 43.5 % sequence identity to the SRP and affiliated SOB Apr lineage II sequences), the oxidative-operating AprBA proteins of lineage II were DsrAB-discordantly placed in closer affiliation with the reductive-operating enzymes of the SRP, which was indicative of LGT events. Within these major Apr lineages, the SOB taxa clustered consistently with their 16S rRNA-gene-based classification into the *Chlorobiaceae* and individual classes of the *Proteobacteria* (see Supplementary Fig. S1, available with the online version of this paper) (Cavanaugh *et al.*, 2001; Imhoff, 2001a, 2003; Kelly & Wood, 2000; Kuever *et al.*, 2002; Polz *et al.*, 1996; Robertson & Kuenen, 2001). In Apr lineage I, the *Alphaproteobacteria* formed a basal branching group separated into (1) the SAR11 clade, comprising *Pelagibacter ubique* (Giovannoni *et al.*, 2005) and closely related environmental sequences, and (2) the SAR116 clade [environmental sequences derived from shotgun sequencing of Sargasso and Red Sea water samples (Venter *et al.*, 2004; G. Sabehi and O. Beja, unpublished data)]. However, the close relatedness of *Thiobacillus plumbophilus* to the *Alphaproteobacteria* is discordant with its 16S rRNA-gene-based betaproteobacterial position. The second group of Apr lineage I consisted of the *Gammaproteobacteria*, with distinct clusters formed by (1) the symbiotic and related, free-living chemotrophic SOB (e.g. symbionts of *Calyptogena magnifica* and *Bathymodiolus* spp., strain OAII2) that are 16S rRNA-discordantly in close affiliation with the group of betaproteobacterial *Thiobacillus* spp., and (2) the anoxygenic phototrophic *Chromatiaceae*. The Apr-based intrafamily branching order of the *Chromatiaceae* taxa was partially incongruent with the 16S rRNA-gene-based phylogeny (Imhoff, 2001a) and indicated putative LGT events among members of the *Chromatiaceae* (e.g. marine *Thiococcus pfennigii* strains were in closer affiliation with freshwater *Allochromatium* spp. than with salt-requiring *Rhabdochromatium* or halophilic *Halochromatium* spp.). In agreement with its 16S rRNA-gene-based

Table 4. Results of Southern blot assays with radioactively labelled *aprA*-specific probes and genomic DNA of SOB and SRB

Genomic DNA of SOB species (*Eco*RI/*Hin*dIII digestion)	DSM*	Southern blot hybridization results with *aprA*-specific probe†			
		Chlorobium limicola 1855	*Chlorobium clathratiforme* 5477	*Thiocapsa roseopersicina 4210***	*Desulfomicrobium baculatum* 4028
Gammaproteobacteria					
Thiocapsa roseopersicina	217	+‡	+	++‡	+
Thiocapsa roseopersicina	*4210***	+‡	+	++‡	+
Chlorobia					
Chlorobium limicola	245**	−	−	−	−
Chlorobium limicola	248**	−	−	−	−
Chlorobium limicola	1855**	++	+	+	+
Chlorobium luteolum	262**	−	−	−	−
Chlorobium luteolum	273**	−	−	−	−
Deltaproteobacteria					
Desulfomicrobium baculatum	4028	++	+	+	++

*Cultures obtained from culture collection of J. Imhoff (laboratory internal numbers in italic type) are marked with **.
†Quality of hybridization results: −, no hybridization; +, hybridization signal; ++, strong hybridization signal.
‡Two hybridization signals obtained with genomic DNA of reference strain *Thiocapsa roseopersicina* DSM 217 and strain *4210* (culture collection of J. Imhoff).

Table 5. Presence of *sat*, *apr* and *qmo* homologues coding for the dissimilatory ATP sulfurylase, the APS reductase and its putative functionally associated proteins (AprM and QmoABC) in genome sequences of SOB (the genomic arrangement of the genes is indicated by the GenBank accession numbers of the encoded proteins)

Environm. seq., environmental sequence.

SOB species	GenBank accession numbers of genome sequences	Homologues present in the SOB genome*						
		Sat	AprM	AprB	AprA	QmoA	QmoB	QmoC
Chlorobia								
Chlorobium clathratiforme str. BU-1	AAIK01000013	ZP_ 00589416	–	ZP_00589417	ZP_00589418	ZP_00589419	ZP_00589420	ZP_ 00589421
Chlorobium chlorochromatii str. CaD3	NC_007514	YP_379885	–	YP_379884	YP_379883	YP_379882	YP_379881	YP_379880
Chlorobium phaeobacteroides str. BS1	NZ_AAIC01000057, NZ_AAIC01000606, NZ_AAIC01000002	ZP_ 00532500	–	ZP_00532499	ZP_00532499‡	ZP_ 005333969‡	ZP_ 005333969‡	ZP_ 00530754
Chlorobaculum tepidum str. TLS	NC_002932	NP_661756	–	NP_661758	NP_661759	NP_661760	NP_661761	NP_661762
Alphaproteobacteria								
SAR11 clade								
Pelagibacter ubique HTCC1002	NC_007205	–	ZP_01264008	ZP_01264007†	ZP_01264006	–	–	–
Pelagibacter ubique HTCC1062	AACY01002870	–	YP_266255	YP_266256	YP_266257	–	–	–
Environm. seq. IBEA_CTG_2004608	AACY01050991	–	EAK63329‡	EAK63329	EAK63328	–	–	–
Environm. seq. IBEA_CTG_2148592	AACY01000596	–	EAJ52712‡	EAJ52712	EAJ52711	–	–	–
Environm. seq. IBEA_CTG_1977812	AACY01023675	–	EAK69056‡	EAK69056	EAK69055‡	–	–	–
Environm. seq. IBEA_CTG_2104149	AACY01055405	–	EAK12872‡	EAK12872	EAK12873	–	–	–
Environm. seq. IBEA_CTG_2157860	AACY01021098	–	–	EAJ42062	EAJ42063	–	–	–
Environm. seq. IBEA_CTG_2023371	NC_007205	EAK19071	EAK19072	EAK19073	EAK19074	–	–	–
Environm. seq. IBEA_CTG_2151824	AACY01077644	–	EAI88884	EAI88883	EAI88882	–	–	–
Environm. seq. IBEA_CTG_2037797	AACY01013231	–	EAK37254	EAK37255	EAK37256	–	–	–
Environm. seq. IBEA_CTG_2149712	AACY01043919	–	EAJ68379	EAJ68380	EAJ68381	–	–	–
Environm. seq. IBEA_CTG_2157518	AACY01120224	–	EAH91106	EAH91107	EAH91108	–	–	–
Environm. seq. IBEA_CTG_2150375	AACY01003082	–	EAK62842	EAK62843	EAK62844	–	–	–
Environm. seq. IBEA_CTG_2126577	AACY01037195	–	EAJ82119	EAJ82118	EAJ82117	–	–	–
Environm. seq. IBEA_CTG_2151480	AACY01061995	–	EAJ26240†	EAJ26241	EAJ26242	–	–	–
SAR116 clade								
Uncultured bacterium EBAC2C11	AY744399	AAV31643	AAV31644	AAV31645	AAV31646	–	–	–
Environm. seq. IBEA_CTG_2159535	AACY01013150	–	EAK37449	EAK37448	EAK37447‡	–	–	–

Table 5. cont.

SOB species	GenBank accession numbers of genome sequences	Homologues present in the SOB genome*						
		Sat	AprM	AprB	AprA	QmoA	QmoB	QmoC
Betaproteobacteria								
Thiobacillus denitrificans ATCC 25259	NC_007404	YP_314632	YP_316042	YP_314631/ YP_316041	YP_314630/ YP_316040	YP_315406	YP_315405	–
Gammaproteobacteria								
Allochromatium vinosum	U84759§	AAC23622	AAK16200	AAC23620	AAC23621	–	–	–
Olavius algarvensis Gamma-3 symbiont	AASZ_01000200	+	+	+	+	–	–	–
Olavius algarvensis Gamma-1 symbiont	AASZ_01004101, AASZ_01004100, AASZ_01000461	ND	–	+‡	+‡	+	+	–
Endoriftia persephone	AASF_01001200, AASF_01001506, AASF_01002103, AASF_01001775, AASF_01001739	+‡	–	+	+	+‡	+‡	–
Cdt. Ruthia magnifica str. CM	NC_008610	YP_903355	YP_903356	YP_903357	YP_903358			

*The presence of protein encoding *sat*, *aprBA*, *aprM* and *qmoABC* homologues in the SOB genome is indicated by the GenBank accession number of the respective protein (or by + for the metagenomic sequences of the gammaproteobacterial symbiont of *Olavius algarvensis*).
†Incorrect gene annotation by automated protein coding gene prediction program in genome sequence.
‡Incomplete gene sequences obtained from unfinished genome/environmental metagenome sequencing projects.
§Partial genome sequence obtained by cloning experiments.

phylogenetic assignment as a member of the genus *Allochromatium*, the AprBA-based trees confirmed the incorrect classification of SOB strain DSM 214 as a *Thiocystis violacea* subspecies.

The *dsrAB*-discordant branching position of Apr lineage II in the Apr trees points to putative lateral transfer of *apr* genes among SRP and SOB. This lineage comprised the proteins of the green sulfur bacteria and several members of the *Chromatiaceae*, *Thiobacillus* and invertebrate symbionts. While some of these beta- and gammaproteobacterial SOB harbour a second *apr* gene locus that codes for the putative resident, 'authentic' homologue (Apr lineage I), the majority exclusively possess *aprBA* genes of the SRP-related Apr lineage II. In this lineage, the green sulfur bacteria formed a monophyletic group (sequence identity >80.9 %) in closer affiliation with the reductive-operating Apr of the *Thermodesulfovibrio–Desulfobacca* cluster than with the other oxidative-acting APS reductases of the proteobacterial SOB. The latter group diverged in agreement with the 16S rRNA phylogeny into (1) the chemotrophic betaproteobacterial SOB (*Thiobacillus*), and (2) the chemo- and phototrophic gammaproteobacterial SOB (*Thiothrix*, symbionts from *Riftia*, *Inanidrilus* and *Ifremeria* and seven *Chromatiaceae* species). However, the AprBA-based basal branching of the phototrophic Fe(II)-oxidizing *Thiodictyon* sp. strain F4 in closest affiliation with the *Thiobacillus* spp. was not consistent with its 16S rRNA-gene-based classification (Ehrenreich & Widdel, 1994).

Additional evidence for LGT of *aprBA*

The presence of indels at identical positions in the AprBA alignments provides additional evidence for LGT events. In addition, the sequences were checked for recent LGT events by identification of atypical sequence characteristics (Lawrence & Ochman, 1997), e.g. significant deviations in mol% G+C content and codon usage between the LGT-derived gene and respective SOB genome, as well as the corresponding *dsrAB* genes.

The separate phylogenetic position of Apr lineage I was confirmed by the presence of nine unique indels in the sequences of both subunits (see grey-shaded boxes in AprBA alignment, Supplementary Fig. S2 available with the online version of this paper). The separation into the alphaproteobacterial and the beta/gammaproteobacterial SOB group in the Apr lineage I was stressed by single, group-specific indels, whereas the 16S rRNA-discordant

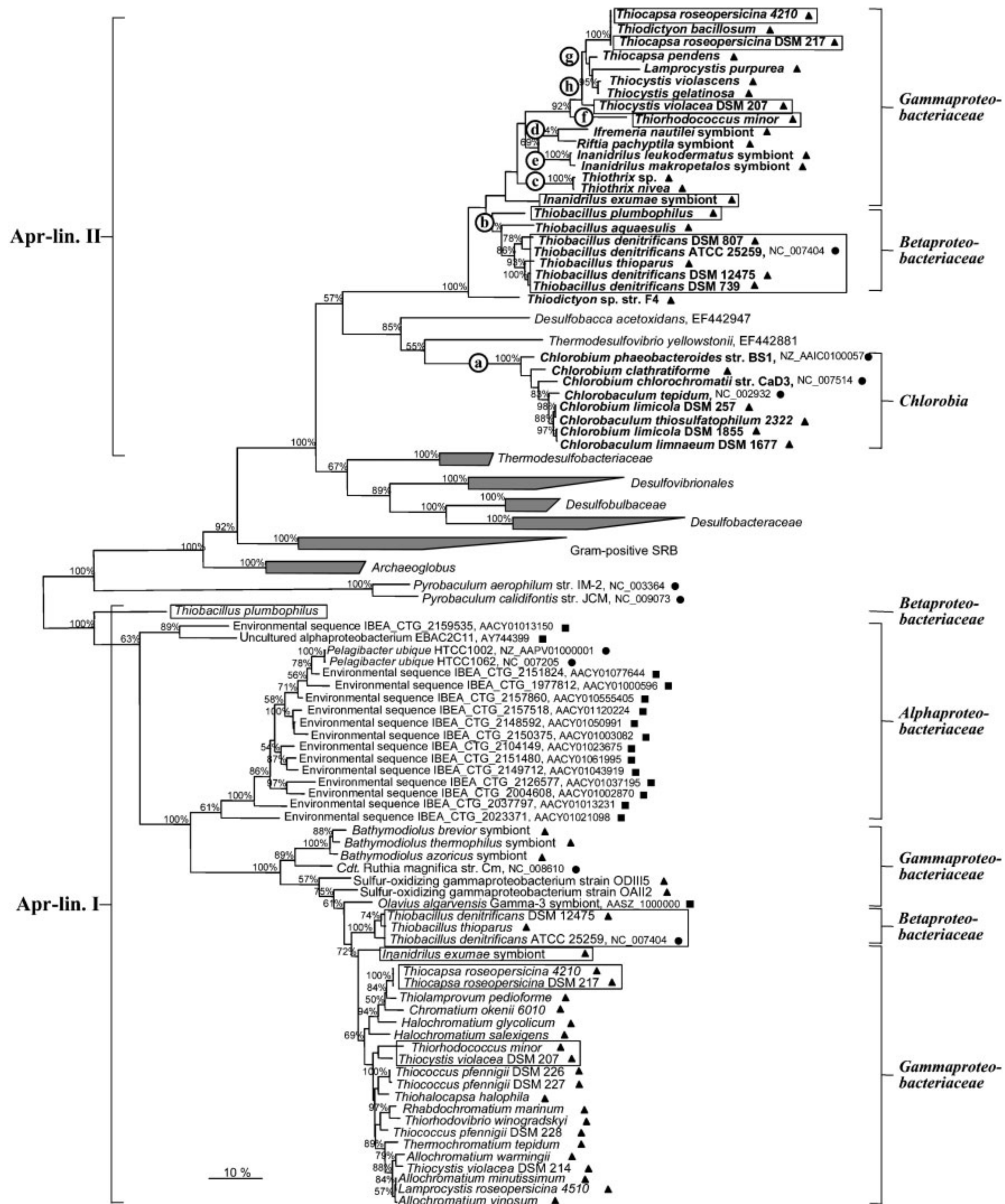

Fig. 1. Phylogenetic tree based on comparative analysis of 379 amino acid positions of 104 AprBA sequences from investigated SOB, including full-length AprBA sequences retrieved from public databases of SOB (genomic, metagenomic and PCR analysis-derived sequences are indicated by ●, ■ and ▲, respectively) and selected SRP. The tree was inferred using the maximum-likelihood method. The DsrAB-congruent, presumably 'authentic' AprBA lineage I (Apr-lin. I) was used as the outgroup reference. SOB with DsrAB-discordant, putative laterally transferred *aprBA* genes are in bold type (Apr lineage II; Apr-lin. II), while the reference strains with two *apr* copies are indicated with boxes. Proposed LGT events are indicated by letters (a–h). The taxonomic classifications of investigated SOB based on the 16S rRNA gene phylogeny are indicated. The scale bar corresponds to 10 % estimated sequence divergence. Cdt., Candidatus; str., strain.

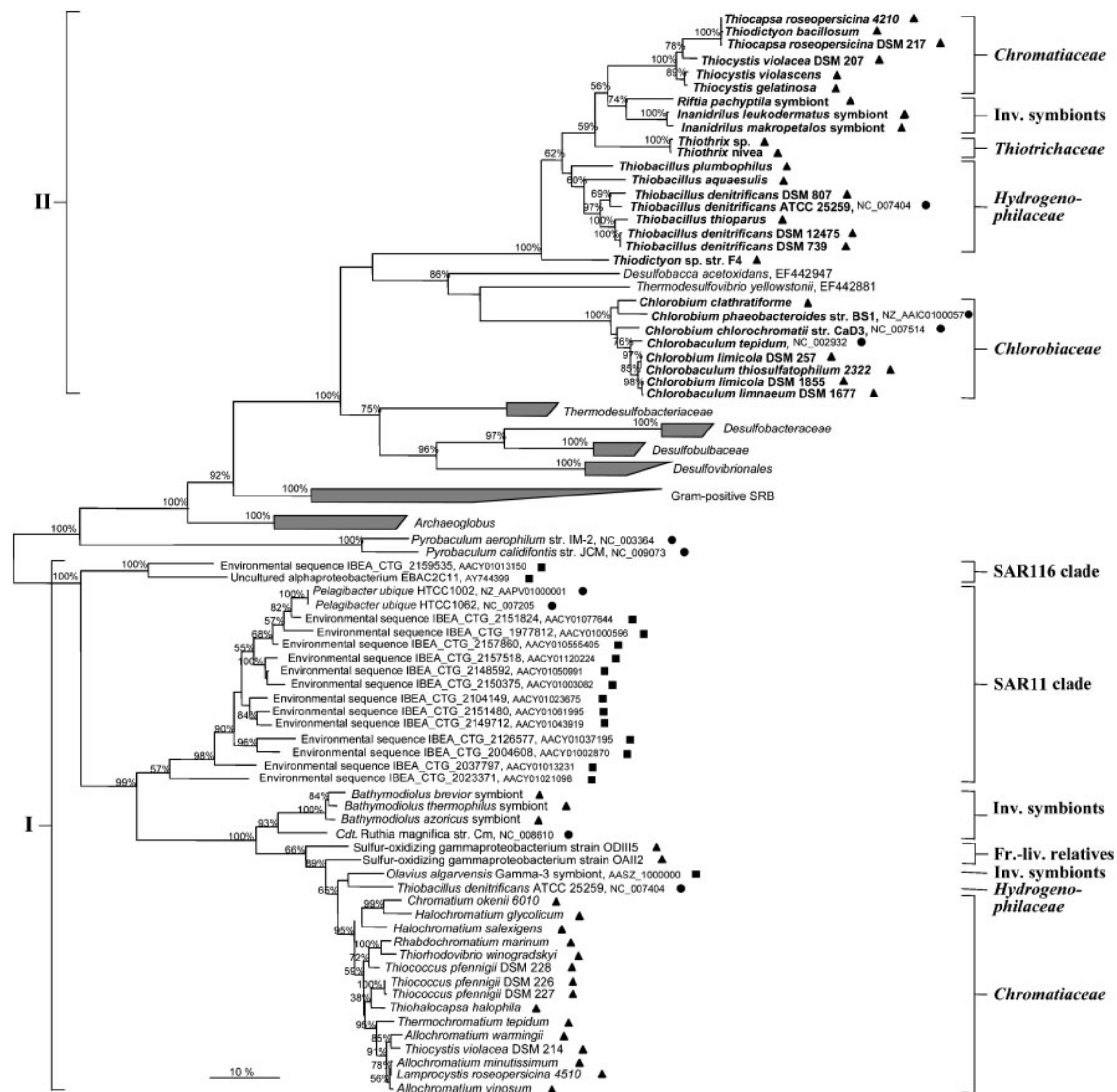

Fig. 2. Phylogenetic tree based on comparative analysis of 701 amino acid positions of 89 AprBA sequences from investigated SOB, including full-length AprBA sequences retrieved from public databases of SOB (genomic, metagenomic and PCR analysis-derived sequences are indicated by ●, ■ and ▲, respectively) and selected SRP. The tree was inferred using the maximum-likelihood method. The DsrAB-congruent, presumably 'authentic' AprBA lineage I was used as the outgroup reference. SOB with DsrAB-discordant, putative laterally transferred *aprBA* genes are in bold type (Apr lineage II). The taxonomic classifications of investigated SOB based on the 16S rRNA gene phylogeny are indicated. The scale bar corresponds to 10 % estimated sequence divergence. Cdt., Candidatus; str., strain; Inv., invertebrate; Fr.-liv., free-living.

branching positions of *Thiobacillus plumbophilus* and symbionts of *Bathymodiolus* spp. in the AprBA trees were supported by group-intermixed indels and further unique insertions in each subunit sequence. The affiliation of *Chlorobiaceae* and *Thermodesulfovibrio* AprBA proteins was confirmed by the presence of four shared indels, while their phylogenetic separation from the proteobacterial Apr sequences in Apr lineage II was proven by the existence of five unique indels. In addition, the 16S rRNA-discordant closest affiliation of the *Thiodictyon* sp. strain F4 with the investigated chemotrophic SOB of the *Beta*- and *Gammaproteobacteria* was supported by three shared indels. The compositional similarity of the LGT-derived *aprBA* sequences of Apr lineage II to the respective recipient genomes points to an ancient timing of all inferred LGTs that affected the investigated SOB. This is also stressed by the strong congruence of the mol% G+C content and codon usage of the corresponding *aprBA* sequences of those SOB that contain two *apr* gene loci, despite their distant relationship.

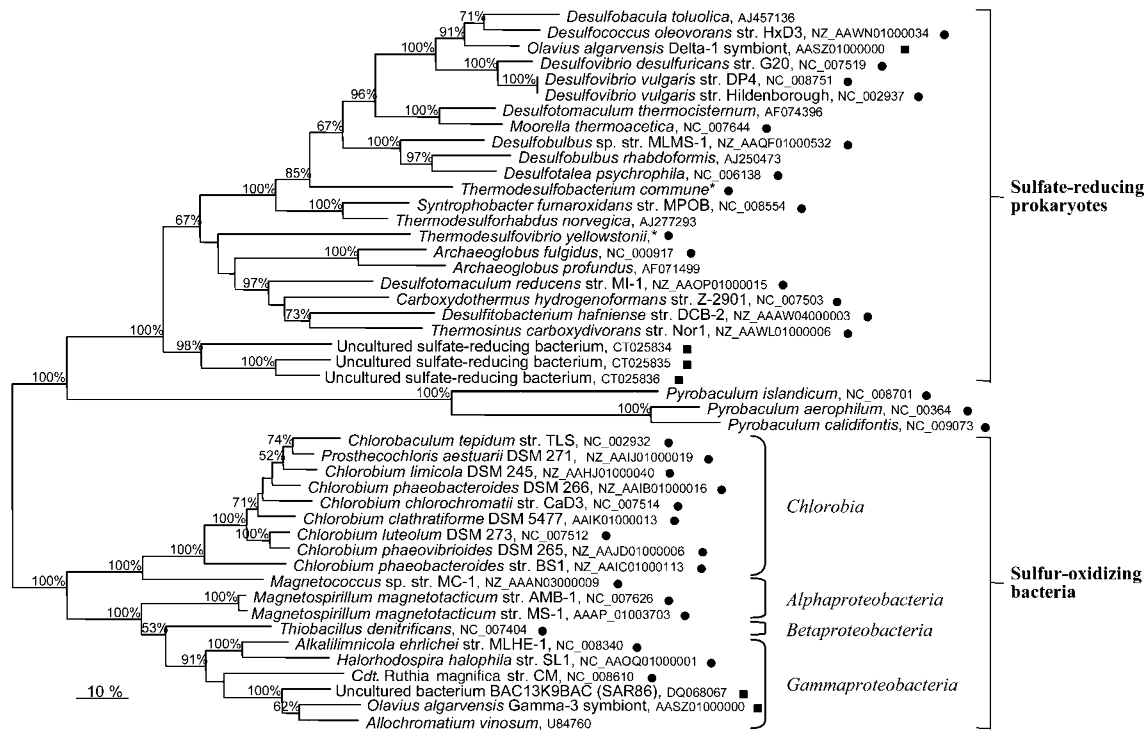

Fig. 3. Phylogenetic tree based on comparative analysis of 740 amino acid positions of 46 DsrAB sequences from full-length sequences retrieved from public databases of SOB and SRP (genomic and metagenomic sequences are indicated by ● and ■, respectively). The tree was inferred using the maximum-likelihood method. The DsrAB lineage of SOB was used as the outgroup reference. The taxonomic classifications of SOB based on the 16S rRNA gene phylogeny are indicated. The scale bar corresponds to 10 % estimated sequence divergence. Cdt., Candidatus; str., strain.

Genomic arrangement of *aprBA* and functionally associated genes in SOB

Interestingly, the phylogenetic divergence of the oxidative-operating APS reductases into two distantly related lineages is reflected in the deviating presence and arrangement of *sat*, *aprM*, *qmoABC* and heterodisulfide reductase-encoding (*hdr*) genes and other hypothetical proteins (UPF0153 and COG1576) in the SOB genomes (see Fig. 4, Table 5). The presumably LGT-unaffected, 'authentic' *aprBA* gene loci (Apr lineage I) of the photo- and chemotrophic *Proteobacteria* are always preceded by and co-transcribed with an *aprM* homologue which is not found in the genomes of SRP, *Chlorobiaceae*, *Endoriftia persephone* or *Olavius algarvensis* Gamma-1 symbiont, or adjacent to the LGT-affected *apr* gene locus II from *Thiobacillus denitrificans.* The native electron acceptor and transfer mechanism to the electron transport chain is unknown for the APS reductases of Apr lineage I. However, comparative sequence analysis predicts the protein AprM to contain five transmembrane helices with no sequence similarity to any currently known conserved domain or cofactor binding site in the databases. An essential structural function of AprM as a membrane anchor that allows the spatial and functional association of this type of oxidative APS reductase with the membrane has been postulated (Meyer & Kuever, 2007).

In accordance with their affiliated AprBA proteins, the *sat-aprBA-qmoABC* genomic arrangement of the *Chlorobiaceae* is nearly identical to the gene organization of *Thermodesulfovibrio yellowstonii* (Fig. 4). The additional ORF located downstream of the *sat* gene in the genomes of thermophilic SRP is missing in the *Chlorobiaceae*; however, unusually long intergenic distances separate the *sat* and *aprB* genes in the latter, indicating the potential loss of this ORF after the transfer event. Interestingly, the gene composition and arrangement of the presumably LGT-derived *apr* gene loci from *Olavius algarvensis* Gamma-1 symbiont, *Endoriftia persephone* and *Thiobacillus denitrificans* are more similar to the SRP than to the *Chlorobiaceae.* The *qmo* operons are truncated to a *qmoAB* gene locus (compare to *Desulfotomaculum reducens* and *Syntrophobacter fumaroxidans*, Fig. 4), but are located in a genomic position distant to that of the *sat–aprBA* operon. A separately transcribed *qmoC* homologue could not be identified; however, *hdrC* and *hdrB* homologues (coding for functional subunits of the heterodisulfide reductase) are present near the *qmoAB* of *Thiobacillus denitrificans*, *Olavius algarvensis* Gamma-1 symbiont and *Endoriftia*

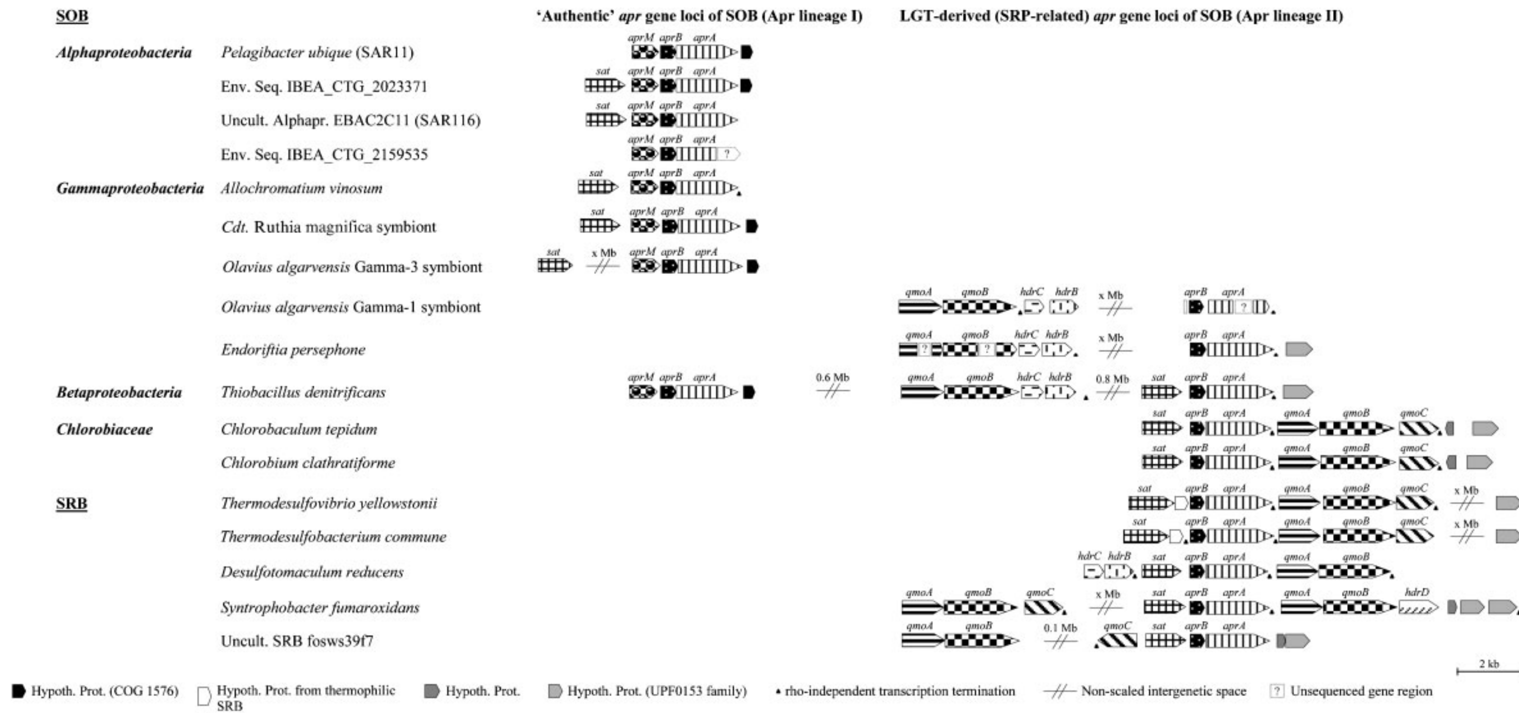

Fig. 4. Diagrammatic comparison of the genomic organization of the *sat*, *apr* and *qmo* genes in selected SOB and SRP (operon structure prediction by Softberry) in support of the inferred LGTs of the entire gene locus from SRP to certain SOB lineages. ORFs were designated based on BLASTX search results with significant homology scores. Loci: *sat*, dissimilatory ATP sulfurylase; *aprA* and *aprB*, α and β subunits of the dissimilatory APS reductase; *aprM*, putative transmembrane protein; *qmoA*, *qmoB* and *qmoC*, subunits of the putative APS : menaquinone oxidoreductase; *hdrB*, *hdrC* and *hdrD*, subunits of heterodisulfide reductase. Abbreviations: Cdt., Candidatus; Env. Seq., environmental sequence; Uncult. Alphapr., uncultured alphaproteobacterium; Hypoth. Prot., hypothetical protein.

persephone (see also *Desulfotomaculum reducens* and *Syntrophobacter fumaroxidans*, Fig. 4). Interestingly, the *qmoAB*, *hdrC* and *hdrB* genes of *Thiobacillus denitrificans* and *Endoriftia persephone* have been predicted to be co-transcribed. This might indicate that the electron transfer from cytoplasmic sulfite oxidation to the membrane quinone pool might be mediated by an SRP-like linking mechanism (Pires *et al.*, 2003) in the Apr lineage II protein-containing SOB.

DISCUSSION

Distribution of *apr* genes among anoxygenic phototrophic SOB

In *Allochromatium vinosum*, the oxidation of intermediately stored sulfur globules has been demonstrated to depend on the activity of the reverse-acting sulfite reductase and membrane-spanning DsrMKJOP complex (Dahl *et al.*, 2005; Pott & Dahl, 1998; Sander *et al.*, 2006). In support of a general, essential role in the oxidative sulfur metabolism of the *Chlorobiaceae*, *Chromatiaceae* and *Ectothiorhodospiraceae* (1) the *dsrAB* and *dsrMKJOP* genes are present in all currently sequenced genomes from representatives of the previous SOB lineages (Dahl *et al.*, 2005; Sabehi *et al.*, 2005; Sander *et al.*, 2006) (see also Supplementary Table S1 available with the online version of this paper), and (2) the existence of *dsr* operons has been confirmed by Southern blot assays in several further members (Dahl *et al.*, 1999). The subsequent oxidation of sulfite can be mediated by either the reverse-operating APS reductase (Apr) or SAOR (Brune, 1995; Brüser *et al.*, 2000; Kappler & Dahl, 2001). Although the reverse-acting Apr has been demonstrated to be constitutively expressed, an essential *in vivo* role for this enzyme in the sulfur metabolism of *Allochromatium vinosum* has recently been questioned (Dahl, 1996; Sanchez *et al.*, 2001). In support of the former results, the PCR-based analysis of this study indicated a generally restricted distribution of the dissimilatory APS reductase-encoding genes among the investigated anoxygenic phototrophic SOB (Table 1). While the presence of *apr* genes could be proven for most *Chromatiaceae* spp. (except marine *Isochromatium* and *Marichromatium*), they are absent in the investigated *Ectothiorhodospiraceae* species and restricted in the *Chlorobiaceae* to members of subclusters 3 and 4b (Imhoff, 2003). Generally, the results of our molecular analysis are in agreement with those of earlier enzyme activity studies (Brune, 1995; Dahl & Trüper, 1994; Trüper & Fischer, 1982); however, the reported APS reductase activity in cell extracts of *Chlorobium limicola* DSM 245, '*Chlorobium vibrioforme* f. *thiosulfatophilum*' NCIB 8346 (*Chlorobaculum parvum 2352* in Table 1) and *Chlorobium phaeovibrioides* DSM 270 (Brune, 1995; Dahl & Trüper, 1994) are not supported. Because all available AprBA sequences of green sulfur bacteria are highly similar in sequence (>80.9 % sequence identity) and possess complementary primer target sites, amplification failure can be excluded. In addition, the proposed absence of *apr* genes of several *Chlorobiaceae* species of subcluster 2b and 3b was confirmed by Southern blot analysis using an *aprA* probe that encompassed the strictly conserved gene region coding for the APS/ sulfite binding site (see hybridization results with *Desulfomicrobium baculatum*, Table 4). Therefore, the existence of *apr* genes that encode functional enzymes could be excluded for the investigated reference strains. Further evidence for the correctness of our PCR results is given by the consistent negative BLAST search results for the currently sequenced genomes of *C. limicola* DSM 245, *Chlorobium luteolum* DSM 273 and *Chlorobium ferrooxidans*.

Growth experiments with *aprBA*-deficient strains of *Allochromatium vinosum* showed that the loss of reverse APS reductase activity had no effect on the specific growth rate and sulfite-oxidizing ability. The sulfite oxidation has been calculated to rely to 69–100 % on the activity of the coexisting SAOR under photolithoautotrophic conditions (Sanchez *et al.*, 2001). Because all green and purple sulfur bacteria investigated in this study are capable of complete sulfide/sulfur oxidation to sulfate, the observed lack of *apr* genes in most examined species might be indirect evidence for the presence and functional importance of the AMP-independent pathway in the oxidative sulfur metabolism of SOB. Indeed, the existence of an SAOR has been established by enzyme assays for several representatives that do not possess the APS pathway, e.g. *Marichromatium* spp. (Trüper & Fischer, 1982). The SAOR of *Allochromatium vinosum* has been suggested to be a molybdenum-containing protein (Dahl, 1996), like the well-documented enzyme of *Starkeya novella*, SorAB (Kappler & Dahl, 2001; Kappler & Bailey, 2005). With regard to its functional association with the essential reverse sulfite reductase (Pott & Dahl, 1998), a cytoplasmically localized SAOR is postulated for the anoxygenic phototrophic SOB. Indeed, periplasmic SorAB-encoding homologues could not be identified in the currently sequenced genomes of *Chlorobiaceae* and *Ectothiorhodospiraceae*. The presence of an additional sulfite-oxidation pathway via APS-reductase (and ATP sulfurylase) besides the SAOR would allow molybdenum-independent sulfite oxidation and might be a selective advantage under certain photolithotrophic conditions (e.g. allowing a greater supply of reducing equivalents at saturating irradiances), or during chemolithoautotrophic growth (observed for several *Chromatiaceae*; Imhoff, 2001a).

In accordance with earlier results based on enzyme assays (Brune, 1995; Friedrich, 1998; Trüper & Fischer, 1982), *apr* genes were not detected in the investigated phototrophic *Alphaproteobacteria* (Table 1). Indeed, their representatives have been reported to prefer a photoorganoheterotrophic mode of growth and to vary considerably in their ability to use reduced sulfur compounds as photosynthetic electron donors (Brune, 1995; Imhoff, 2001b). If utilized, sulfide is primarily converted (by SQR) to elemental sulfur instead

of sulfate (Griesbeck *et al.*, 2000). The absence of *apr* and *dsr* genes in their genomes confirms their use of a different enzymic sulfur oxidation system compared to the cytoplasmic pathway of the previously discussed SOB lineages. Interestingly, the only members of the *Alphaproteobacteria* that harbour *apr* genes in their genomes (Table 5) are *Pelagibacter ubique* subsp. (SAR11) and an uncultured putative SOB of the SAR116 clade. Since they lack *sat* and *dsrAB* genes, a dissimilatory function of the APS reductase in their sulfur metabolism can be excluded. The reverse-acting APS reductase might primarily be used for detoxification of sulfite to prevent its cytoplasmic accumulation during the degradation of organic sulfur compounds. Indeed, members of the SAR11 clade have been demonstrated to dominate the assimilation of dissolved dimethylsulfoniopropionate (DMSP) in the surface ocean (Malmstrom *et al.*, 2004).

Distribution of *apr* genes among chemotrophic SOB

Diverse enzymic systems have been suggested to be involved in the aerobic oxidation of sulfide/sulfur to sulfate in the phylogenetically divergent chemotrophic SOB, comprising primarily the periplasmic enzymes SQR, sulfur dioxygenase (SDR) and SAOR (Brüser *et al.*, 2000; Friedrich, 1998; Griesbeck *et al.*, 2000; Kappler & Dahl, 2001; Kelly, 1999; Rohwerder & Sand, 2003; Suzuki, 1994; Takai *et al.*, 2005; Teske & Nelson, 2004; Theissen *et al.*, 2003). Consistently, this study revealed the *apr* genes to be restricted among the chemotrophic SOB to representatives of *Thiobacillus*, *Thiothrix* and invertebrate symbionts, including some of their free-living relatives, in accordance with earlier enzyme activity studies (Friedrich, 1998; Nelson & Fisher, 1995; Odintsova *et al.*, 1993; Taylor, 1994). Furthermore, APS reductase activity has been found in obligate chemolithoautotrophic, marine *Beggiatoa* subspecies but seems to be absent in mixo- or even heterotrophic freshwater strains (Teske & Nelson, 2004) as confirmed by this molecular analysis. In congruence to the results of this study (Table 1), no APS reductase has been reported to be involved in the oxidative sulfur metabolism of *Thiomicrospira* spp. (Nelson & Fisher, 1995), *Sulfurimonas denitrificans* (Takai *et al.*, 2005) and acidophilic SOB (Brüser *et al.*, 2000). The sulfur metabolism of *Aquaspirillum*, *Macromonas* and *Spirochaeta* spp. (Dubinina *et al.*, 2004; LaRiviere & Schmidt, 2001) has not yet been investigated by enzyme studies.

Interestingly, most chemotrophic SOB that possess *apr* genes are facultative anaerobes with the ability to switch between aerobic and anaerobic modes of growth (Brüser *et al.*, 2000; Cavanaugh *et al.*, 2001; Kuever *et al.*, 2002; Nelson & Fisher, 1995; Robertson & Kuenen, 2001). The cytoplasmic enzyme system (Dsr, Apr and Sat/APAT) might operate in these chemotrophic SOB in a manner analogous to that of the anoxygenic phototropic *Chromatiaceae* (and *Chlorobiaceae*), with significant energetic advantages for chemothioautotrophic growth under oxygen limitation (electron transport phosphorylation-coupled sulfur oxidation; electrons enter the electron transport chain at the energetic level of quinones with a higher number of coupling sites available to drive ATP synthesis and lower energy demand for reverse electron flow; substrate phosphorylation-coupled sulfite oxidation). Indeed, the expression and involvement of Dsr, Apr and Sat in the sulfur oxidation process has been confirmed for anaerobically grown cultures of *Thiobacillus denitrificans* (Beller *et al.*, 2006; Schedel & Trüper, 1980) and the endosymbiont of *Riftia pachyptila* (Markert *et al.*, 2007). A general involvement of the latter pathway was suggested by Kelly (1999), based on the greater molar growth yields of *Thiobacillus denitrificans* compared to *Sulfurimonas denitrificans* even under aerobic conditions. In contrast, the involvement of an extracytoplasmic sulfur-oxidation pathway (via SQR, SDR and SAOR) might in principle be more advantageous (no requirement for energy-demanding sulfur compound permease systems; no cytoplasmic accumulation of toxic substrates or products; no acidification of the cytoplasm while the extracytoplasmic localization of substrate oxidation contributes to the proton gradient).

The enzymes of the archaeal sulfur-oxidation pathway (e.g. the unique sulfur oxygenase reductase) represent convergent evolutionary lines compared to the bacterial proteins (Brüser *et al.*, 2000; Kletzin *et al.*, 2004). Based on enzyme activity assays, an involvement of the reverse APS reductase in the sulfite oxidation of *Acidianus ambivalens* has been postulated, despite high levels of non-enzymic background (Zimmermann *et al.*, 1999). In contrast, the results of our molecular study do not support its involvement in the oxidative sulfur metabolism of *Sulfolobales* representatives, including *Acidianus ambivalens*.

Phylogeny of dissimilatory APS reductase from SOB indicates lateral transfer of *aprBA* genes between SRP and SOB

The DsrAB phylogeny presents the oxidative-operating sulfite reductases of SOB as monophyletic and distantly related to the reductive enzyme type of the SRP (Fig. 3). In contrast, the reverse APS reductases of photo- and chemotrophic SOB appear to have diverged into two distinct phylogenetic clusters, comprising the DsrAB-congruent Apr lineage I and the DsrAB-discordant Apr lineage II (Figs 1 and 2). Accumulating experimental evidence confirms the postulated essential function of the reverse sulfite reductase (including the DsrMKJOP transmembrane redox complex) in the dissimilatory sulfur metabolism of SOB (Beller *et al.*, 2006; Dahl *et al.*, 1999, 2005; Pott & Dahl, 1998; Sander *et al.*, 2006) (also reflected by the ubiquitous presence of *dsr* homologues in genomes; Supplementary Table S1). Thus, the DsrAB-congruent phylogenetic placement most likely represents the 'authentic', LGT-unaffected position of SOB in the Apr tree. The most reasonable explanation for the presence of the

SRP-related *apr* genes (Apr lineage II) in *Chlorobiaceae* and several *Beta*- and *Gammaproteobacteria* (in some representatives in addition to the 'authentic' *apr* gene locus) is the occurrence of multiple LGT events between SRP and SOB. In analogy, the SRP-affiliated *dsrMKJOP* genes of green sulfur bacteria have recently been postulated to have been acquired via lateral transfer from an unknown SRP donor (Sander *et al.*, 2006). Because the AprBA proteins of *Chlorobiaceae* are more closely related to the *Thermodesulfovibrio–Desulfobacca* cluster than to the *Beta*- and *Gammaproteobacteria* (Figs 1 and 2), these SOB groups will have received their LGT-derived *apr* genes independently. Although direct SRP donor lineages are not apparent, putative thermophilic strains might have transferred their *apr* genes concurrently to ancestral strains of the aforementioned groups. A direct lateral transfer of the entire gene locus from the *Thermodesulfovibrio* donor lineage to the *Chlorobiaceae* is supported by (1) the similarity of their indel pattern (Supplementary Fig. S2 available with the online version of this paper), (2) the congruent arrangement of the *sat*, *apr* and *qmoABC* genes in the genomes (Fig. 4), as well as (3) the Apr-consistent topologies of the Sat and QmoABC phylogenetic trees (not shown). The restricted distribution of the *apr* genes among the green sulfur bacteria might indicate that the LGT occurred after their diversification into marine and freshwater strains (Imhoff, 2003). The LGT-derived character of proteobacterial proteins of Apr lineage II is supported by the fact that the SRP-affiliated proteins are restricted to a few beta- and gammaproteobacterial genera and mainly comprise species that harbour two *aprBA* gene loci of which one codes for a presumably 'authentic' protein (Tables 1 and 3). Additional support for an SRP origin of the Apr lineage II proteins in *Proteobacteria* is given by (1) the similar gene locus composition and organization in the betaproteobacterial *Thiobacillus denitrificans* and *Olavius algarvensis* Gamma-1 symbiont compared to the SRP (Table 5 and Fig. 4), and (2) their close relationship on the basis of the QmoA/B proteins (tree not shown). The *apr* genes might have been received via at least seven independent LGT events by the ancestors of *Thiobacillus*, *Thiothrix*, the invertebrate Gamma-1 and -4 symbionts, the *Thiocapsa–Thiodictyon–Lamprocystis* cluster, *Thiocystis* and *Thiorhodococcus*. After diversification, the resident, 'authentic' *apr* gene might have been replaced in several species by its SRP-affiliated xenologue (xenologous gene displacement), causing the observed polyphyly of the *Beta*- and *Gammaproteobacteria* in the Apr-based tree. Indeed, close associations and synergistic interactions between those SOB species that harbour SRP-related *aprBA* genes and SRP have been reported from various habitats and even in invertebrate tissues (Bright & Giere, 2005; Imhoff, 2001a; Markert *et al.*, 2007; Overmann & Garcia-Pichel, 2001; Tonolla *et al.*, 2004; Visscher *et al.*, 1992). Microbial biofilms and mats have been demonstrated to be especially hot spots for LGT involving different physiological groups of prokaryotes (Molin & Tolker-Nielsen, 2003; Sorensen *et al.*, 2005).

Notably, some inferred relationships in the Apr lineage I subtree are discordant with the 16S rRNA-gene-based species phylogeny and indicate the occurrence of putative lateral transfers even among the SOB, e.g. the SAR116-affiliated AprBA proteins of *Thiobacillus plumbophilus*. Furthermore, the correlation of the salt requirement-correlated *Chromatiaceae* classification is not reflected in the Apr-based tree (Imhoff, 2001a). Interestingly, the Apr-based intrafamily branching order is confirmed by the sulfate thiohydrolase (SoxB)-based phylogeny (Meyer *et al.*, 2007). In contrast to the 16S rRNA-gene-based phylogeny (Cavanaugh *et al.*, 2001), the AprBA proteins of *Bathymodiolus* spp. and *Calyptogena magnifica* symbionts form a basal-branching group in the Apr lineage I distinct from their closest relatives *Olavius algarvensis* Gamma-1 symbiont, and SOB strains OAII2 and ODIII5 (Kuever *et al.*, 2002). This might be a result of their vertical mode of transmission (Cary & Giovannoni, 1993; Hurtado *et al.*, 2003), which disconnects the mussel- and bivalve-inhabiting SOB populations from their free-living counterparts and causes elevated evolutionary rates and genetic drift in the symbiotic population by co-adaptation to the host (Peek *et al.*, 1998). The symbiotic bacteria of vestimentiferan tube worms and gastropods that are instead environmentally acquired by their hosts from free-living populations (Bright & Giere, 2005; Cavanaugh *et al.*, 2001; Nelson & Fisher, 1995) have had a higher probability of interspecies gene exchange with the environmental microbial community and harbour xenologous *apr* genes (Fig. 1). A recent 16S rRNA gene analysis of thiotrophic symbionts in *Inanidrilus* spp. (oligochaetes) has revealed the presence of Gamma-4 symbionts in *Inandrilus exumae* (Bergin *et al.*, 2006), which replace the sulfur-oxidizing Gamma-1 symbionts found in other *Inanidrilus* spp. (Blazejak *et al.*, 2006; Bright & Giere, 2005). The distant relationship of the *Inandrilus exumae* Gamma-4 symbiont is reflected in the Apr-based tree and in the presence of two *apr* gene loci that encode proteins of both lineages.

Putative functional importance of SRP-related APS reductases in SOB

The postulated distinct electron-transfer strategies via AprM or Qmo/Hdr are supported by observed differences in the degrees of membrane binding of lineage I and II APS reductases in the photo- and chemotrophic SOB species (Brüser *et al.*, 2000; Dahl & Trüper, 1994; Friedrich, 1998). Interestingly, in *Chromatiaceae* species that contain *apr* genes which code for lineage I proteins (e.g. *Allochromatium vinosum* and *Allochromatium warmingii*), the APS reductases have been reported to be firmly membrane-bound, whereas they have been described as being soluble in those species that harbour the SRP-related *apr* genes encoding the lineage II proteins (e.g. *Thiocapsa roseopersicina*). A functionally important role for the LGT-derived APS reductases in SOB is indicated by the preferential expression of the SRP-related *apr* gene locus compared to the concurrent 'authentic' one in cells of

Thiocapsa roseopersicina (only the soluble APS reductase form is detected in culture; Dahl & Trüper, 1994) and *Thiobacillus denitrificans* (transcriptional analysis; Beller *et al.*, 2006). Recent proteome analysis of *Endoriftia persephone* has confirmed the essential role of the SRP-related APS reductase in the sulfur-dependent energy metabolism of that micro-organism (Markert *et al.*, 2007). Interestingly, *Thiobacillus denitrificans*, *Olavius algarvensis* Gamma-1 symbiont and *Endoriftia persephone* harbour SRP-related *qmoAB* and *hdrCB* homologues in their genomes, indicating the presence of an SRP-related mechanism linking cytoplasmic sulfite oxidation to the membrane quinone pool (Fig. 4). Indeed, *qmoB* has been demonstrated to be expressed in addition to *aprBA* in chemolithoautotrophically growing *Endoriftia persephone* (Markert *et al.*, 2007). By analogy with the postulated interaction of DsrAB and transmembrane complex DsrMKJOP via conserved cysteine residues in DsrC proteins (Dahl *et al.*, 2005; Pires *et al.*, 2006), the SRP-related reverse APS reductase and the QmoAB–HdrCB complex might couple the cytoplasmic sulfite/APS redox process to the electron transport chain by reversible disulfide/thiol interchanges. With regard to the multiple xenologous *aprBA* gene displacements, e.g. in chemotrophic *Thiobacillus* spp. and gammaproteobacterial SOB, the SRP-related APS reductase and electron transfer mechanism might be more advantageous for chemolithotrophic growth. Indeed, the members of the *Chromatiaceae* genera that contain SRP-related *apr* gene loci (Table 1) have been demonstrated to be physiologically versatile, e.g. by their ability to switch between photo- and chemolithoautotrophic lifestyles (Imhoff, 2001a). The two-gene state might be an intermediate in the replacement of the resident essential gene by the LGT-derived foreign homologue. However, the genomic persistence of both gene loci might allow the adaptation of the energy conservation process when growing (1) in dynamic and unstable habitats such as microbial mats with diurnal oscillating sulfide and oxygen concentrations, or (2) in competition with chemotrophic SOB (Imhoff, 2001a; Overmann & Garcia-Pichel, 2001), and might be a selective advantage, especially for non-motile species such as *Thiocapsa roseopersicina* (Imhoff, 2001a).

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