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Riding the sulfur cycle – metabolism of sulfonates and sulfate esters in Gram-negative bacteria

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Abstract

Sulfonates and sulfate esters are widespread in nature, and make up over 95% of the sulfur content of most aerobic soils. Many microorganisms can use sulfonates and sulfate esters as a source of sulfur for growth, even when they are unable to metabolize the carbon skeleton of the compounds. In these organisms, expression of sulfatases and sulfonatases is repressed in the presence of sulfate, in a process mediated by the LysR-type regulator protein CysB, and the corresponding genes therefore constitute an extension of the *cys* regulon. Additional regulator proteins required for sulfonate desulfonation have been identified in *Escherichia coli* (the Cbl protein) and *Pseudomonas putida* (the AsfR protein). Desulfonation of aromatic and aliphatic sulfonates as sulfur sources by aerobic bacteria is oxygen-dependent, carried out by the α-ketoglutarate-dependent taurine dioxygenase, or by one of several $FMNH_2$-dependent monooxygenases. Desulfurization of condensed thiophenes is also $FMNH_2$-dependent, both in the rhodococci and in two Gram-negative species. Bacterial utilization of aromatic sulfate esters is catalyzed by arylsulfatases, most of which are related to human lysosomal sulfatases and contain an active-site formylglycine group that is generated post-translationally. Sulfate-regulated alkylsulfatases, by contrast, are less well characterized. Our increasing knowledge of the sulfur-regulated metabolism of organosulfur compounds suggests applications in practical fields such as biodesulfurization, bioremediation, and optimization of crop sulfur nutrition.

Keywords: Sulfonate; Sulfate ester; Organosulfur metabolism; Sulfur cycle; Sulfatase; Sulfonatase

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1. Introduction

All organisms require sulfur for growth. In bacteria sulfur makes up 0.5–1% of the cell dry weight, and is needed primarily as a component of the amino acids cysteine and methionine. Sulfur also plays an essential role in a variety of enzyme cofactors, including biotin, coenzyme A, coenzyme M, thiamine and lipoic acid, and is critical in many redox processes, both as a building block for iron-sulfur centers and as the redox-active component of disulfide bonds. Sulfur is common in the environment, making up about 0.1% of the earth's crust [1], but much of this material is inaccessible to living organisms. Sulfur for biosynthetic processes is derived from the assimilation of inorganic sulfate by plants and bacteria (animals are unable to synthesize methionine and can synthesize cysteine, the key intermediate in most pathways of sulfur metabolism, only by transsulfurylation from dietary methionine [2]). Cysteine biosynthesis by the sulfate assimilation pathway proceeds by the transport of inorganic sulfate into the cell, its activation by conversion to 3′-phosphoadenosine-5′-phosphosulfate (PAPS), subsequent reduction to sulfite and thence to sulfide, and transfer of this sulfide onto an organic moiety to yield the target molecule cysteine (Fig. 1). The biochemistry and genetics of this process have been extensively characterized in both plants and bacteria, and have been the subject of recent reviews [3–5]. However, perhaps because the growth media traditionally used in the laboratory contain high levels of inorganic sulfate (minimal media contain 1–15 mM sulfate [6]; LB medium contains only about 100–150 μM sulfate, but excess cysteine), cysteine biosynthesis starting from sulfur sources other than sulfate has not been investigated in detail until quite recently. The purpose of this article is to review recent advances in the genetics and biochemistry of how Gram-negative bacteria utilize organosulfur compounds, in particular sulfonates ($R\text{-}SO_3^-$) and sulfate esters ($R\text{-}OSO_3^-$), to provide themselves with cysteine and methionine for growth. It will concentrate on assimilative sulfur metabolism by aerobic bacteria, though in some contexts it has been necessary to include comparisons with anaerobic organisms and with yeasts and other eukaryotes. For a more detailed description of dissimilative organosulfur utilization by sulfate-reducing bacteria and other anaerobes the reader is directed to recent reviews of this subject [7,8]. Sulfur metabolism in yeasts and filamentous fungi has also been reviewed recently [9,10].

Our understanding of the biochemical processes involved in organosulfur utilization by bacteria has leapt forward in recent years, with the characterization of several new sulfur-regulated desulfurizing enzyme systems [11–16]. Additional information has become available through the sequencing of bacterial genomes, 21 of which have been completely sequenced at the time of writing. Where possible, data from these projects have been included in this article. Homologues of many of the genes that are involved in organosulfur metabolism can be identified in other contexts on the chromosomes of other organisms, yielding further information about the substrate flexibility and regulatory complexity of their expression. Conclusions based on this kind of information are necessarily speculative, but may provide useful starting points for future research, and help to put biochemical and physiological flesh on the genetic skeleton supplied by the genome sequences.

Studies of bacterial assimilative organosulfur metabolism are intimately connected with studies of cysteine biosynthesis. Bacterial sulfur assimilation has frequently been assumed to follow the characterized pathway that has been established in enteric bacteria, regulated on a global level as part of the *cys* regulon [5,17]. More recent studies have shown that in non-enteric bacteria, the regulation of cysteine biosynthesis may be somewhat different (e.g. in *Bacillus* [18] or in *Pseudomonas* [19,20]). The initial proteomic study described below suggested that organosulfur metabolism might be controlled by a separate regulatory network to cysteine biosynthesis [21], but by now it has become clear that in most Gram-negative bacteria it is also part of the *cys* regulon, which is much more extensive than previously recognized (as foreseen by Kredich in his review in 1996 [5]).

2. Bacterial responses to sulfate limitation

In the laboratory, bacteria are usually grown either in mineral salts media that provide them with a large excess of sulfur in the form of inorganic sulfate, or in complex media containing not only sulfate but also amino acid sulfur derived from cell hydrolysates. How do they react when they are starved of inorganic sulfate or cysteine? In the absence of these two compounds, bacteria synthesize specific proteins which are required for mobilization of alternative sulfur sources, and for using the last traces of available sulfate. Appropriate sulfate-limited (but not growth-limited) conditions can be provided by supplying the cell with sulfur in organically bound form, e.g. sulfonates, sulfate esters, methionine, sulfamates ($R\text{-}NHSO_3$),

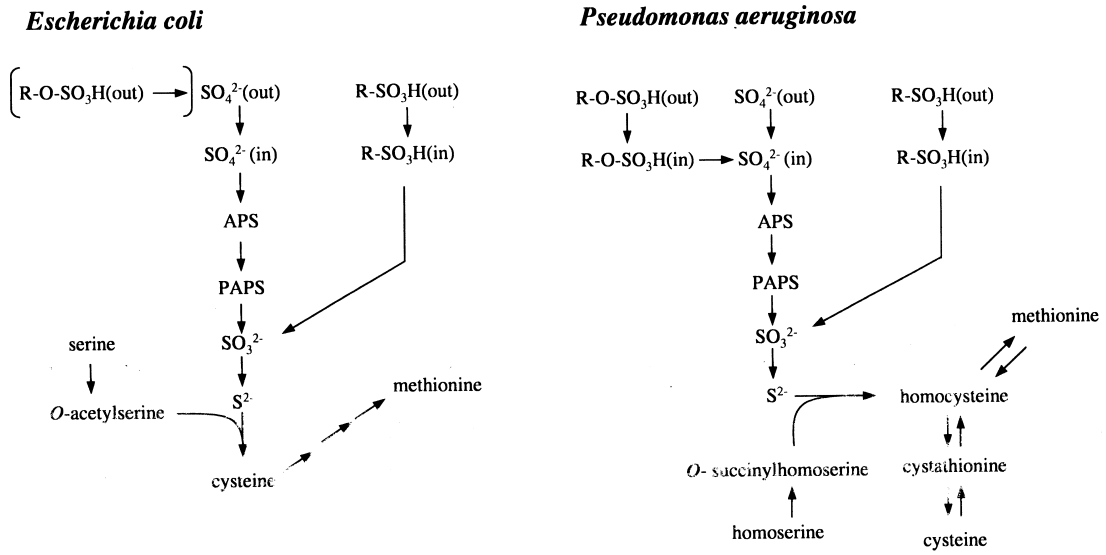


Fig. 1. Cysteine biosynthetic pathways in *E. coli* and *P. aeruginosa*. In enteric bacteria, sulfide is transferred to a serine moiety to give cysteine, whereas in *P. aeruginosa* the acceptor molecule is *O*-succinylhomoserine, yielding homocysteine. Although *E. coli* does not desulfurize sulfate esters, other enteric bacteria do so, using periplasmic sulfatases (square brackets, top left). APS: adenosine-5′-phosphosulfate; PAPS: 3′-phosphoadenosine-5′-phosphosulfate.

organosulfides (R-SS-R′) or thioethers (R-S-R′), in the absence of inorganic sulfate. The cellular response has been examined in several proteomic studies [20–22].

Under sulfate-limited conditions a set of extra proteins was synthesized by several species of bacteria, and identified by differential screening with two-dimensional electrophoretic techniques [20–22]. These so-called sulfate starvation-induced proteins (SSI proteins) are synthesized only in the absence of 'preferred sulfur sources', which vary according to species, but include sulfate, cysteine and thiocyanate. SSI proteins have been found in Gram-positive and Gram-negative species, in soil bacteria and in human pathogens (*Pseudomonas putida, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*). They are not synthesized after heat shock, under phosphorus or nitrogen starvation conditions, or when the cells are grown anaerobically, and therefore appear to constitute a specific response to the absence of sulfate or a related metabolite [20].

The SSI proteins of *E. coli* and *P. aeruginosa* have been characterized by N-terminal sequencing, and by mass spectrometric fingerprinting [20,22–24] (Table 1). The SSI proteins fall into three categories.

(i) They are enzymes and transport systems involved in scavenging and metabolizing alternative sulfur sources from the environment. In this capacity the SSI response can be seen as functionally equivalent to the *pho* response, which governs the utilization of phosphonates and phosphate esters as a response to phosphate limitation in a variety of species [25–28]. In *E. coli* and *P. aeruginosa* these SSI proteins include periplasmic binding proteins for sulfate, cystine and sulfonates, which are anticipated to be involved in sulfur scavenging, and a variety of sulfonatases (Table 1).

(ii) They can be low-sulfur copies of important cellular proteins, in which cysteine or methionine residues which are not critical for function have been replaced by other amino acid residues. Cyanobacteria, for instance, synthesize a second set of phycobilisomal proteins with reduced cysteine/methionine content under sulfate-limited growth conditions [29]. In *P. aeruginosa* a low-sulfur version of the lipoprotein NlpA was found, which may have structural relevance under sulfate-limited growth conditions. Synthesis of several antioxidant proteins was also upregulated during sulfate starvation (Table 1), including alkylhydroperoxide reductase and a thiol-specific antioxidant protein. Synthesis of these proteins may be a response to peroxide intermediates generated as byproducts of sulfonate metabolism (see below), or may have a more direct link to sulfur metabolism, but this has not yet been explored.

(iii) They could be enzymes involved in mobilization of intracellular sulfur storage compounds (the total sulfur content of *E. coli*, for instance, drops by half during sulfur limitation [30], implying that viability is maintained via a redistribution of sulfur within the cell). As yet, no such SSI proteins have been identified. It is interesting that many of the SSI proteins identified in these studies [20,22–24] had previously been undetected, since even in detailed studies such as the *E. coli* proteome analysis carried out by the Neidhardt group, the growth media contained 286 μM sulfate [6], and the SSI proteins were therefore synthesized only at low levels.

The above study in our laboratory was the first to investigate the synthesis of a set of proteins as part of a global sulfate starvation response in bacteria, but a similar global response to limitation for preferred sulfur sources has been recognized in yeast and in filamentous fungi

[9,31,32]. In bacteria, quite a number of enzymes are known whose synthesis is regulated by sulfur supply, primarily those of the cysteine biosynthetic pathway (Fig. 1). The corresponding *cys* genes in enteric bacteria are activated by the CysB protein, and constitute the *cys* regulon. During growth in the presence of excess inorganic sulfate expression of the *cys* genes is reduced to 40–50% of fully derepressed values [5]. This degree of repression is, however, much less than for the SSI proteins detected in our initial study, many of which could not be observed at all in cells grown with sulfate [20–22].

Arylsulfatase is the best characterized bacterial enzyme that is known to be strongly regulated by sulfate starvation, in bacteria, algae [33,34], and filamentous fungi [31,35]. Surprisingly, it was not identified by the differential two-dimensional analysis technique described above, because the amount of arylsulfatase protein synthesized even under fully derepressed conditions was below the detection limit of the method used [11]. Since arylsulfatase activity is strongly upregulated in the absence of sulfate in a variety of bacterial species [21,35], this suggests that sulfate-regulated enzymes may be synthesized at lower levels than other proteins (corresponding to the cell's lower requirement for sulfur, compared to other macronutrients). It is likely, therefore, that further sulfate-regulated proteins still remain undetected.

The data summarized in Table 1 suggest that the bacterial response to sulfate limitation is largely an adaptation that allows the cells to use sulfonates or sulfate esters as an alternative source of sulfur for growth, in the absence of preferred sulfur sources. These are usually sulfate, sulfite, sulfide or cysteine, but methionine is also a preferred sulfur source in fungi [9,31], and thiocyanate represses the SSI response in pseudomonads [21]. Since sulfonates and sulfate esters are not generally accepted to be common classes of compounds, we must next ask why such an adaptation may have evolved, and where sulfonates and sulfate esters occur in nature.

3. Sulfonates and sulfate esters in nature

Plants can only synthesize cysteine from inorganic sulfate. However, in soil environments sulfate often constitutes only 1–5% of the total available sulfur, and it has now been conclusively shown that most of the sulfur present is in organically bound form, either as sulfate esters (over 50% of total sulfur in grassland soils [36]) or as sulfonates (e.g. in forest soils [37]) (Fig. 2). Sulfate for plant nutrition is provided in these systems by sulfur cycling in soil organic matter, catalyzed predominantly by microbial action. Anaerobic soils and sediments also contain considerable quantities of sulfide, due to the action of sulfate-reducing bacteria, and aquatic environments are usually high in inorganic sulfate (both limnic and marine environments – seawater contains an average of 28.7 mM inorganic sulfate [38]). Xenobiotic and naturally occurring sulfonates and sulfate esters available to bacteria as sources of sulfur or carbon are described below.

3.1. Naturally occurring sulfonates

Naturally occurring sulfonates have until quite recently been regarded as few in number, and of minimal importance in the biological sulfur cycle, though they often have important biological functions. They include coenzyme M

Table 1
Sulfate-regulated proteins of *E. coli* and *P. aeruginosa*[a]

Protein	Gene locus	Function	Reference
P. aeruginosa			
PA1	*sbp*	periplasmic sulfate binding protein	[20]
PA2, PA11, PA13	*ssuEADCBF, msuEDC*	alkanesulfonate desulfurization	[20,24]
PA11	*lsfA*	thiol-specific antioxidant	[20,24]
PA9	*tauABCD*	taurine desulfurization	[20,24]
PA7	*nlpA*	lipoprotein	[20,24]
PA4	*atsK*	unknown	[20,24]
PA19	similar to *E. coli fliY*	putative amino acid transport protein	[24]
PA17	similar to *P. putida asfC*	putative periplasmic sulfonate binding protein	[24]
PA14	similar to *E. coli ahpC*	alkylhydroperoxide reductase subunit	[24]
AtsA	*atsA*	arylsulfatase	[11]
AtsRBC	*atsR, atsBC*	transport of sulfate esters	[136]
E. coli			
TauA, TauD	*tauABCD*	taurine desulfurization	[218]
SsuE, SsuD	*ssuEADCB*	alkanesulfonate desulfurization	[22]
Sbp	*sbp*	periplasmic sulfate binding protein	[22]
FliY	*fliY*	periplasmic cystine binding protein	[22]
AhpC	*ahpC*	alkylhydroperoxide reductase subunit	[22]

[a]In *E. coli*, expression of the genes of the *cys* regulon is also repressed during growth in the presence of sulfate [5]. This is presumably also the case in *P. aeruginosa*, though it has not yet been demonstrated. For simplicity, the cysteine biosynthetic genes have been omitted from this table.

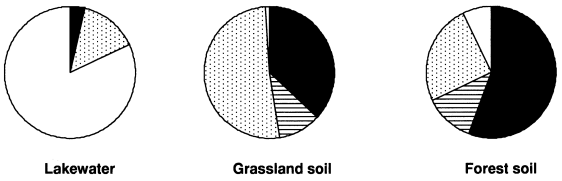


Fig. 2. Sulfur speciation in lakewater [323], and in forest [37] and grassland [36] soils. The sulfur composition is shown as inorganic sulfate (□), sulfate ester sulfur (⋮⋮), amino acid sulfur (⊟), and sulfonate sulfur (■). For lakewater, amino acid and sulfonate sulfur are combined in one fraction.

in methanogenic bacteria [39], taurine in mammals [40], methanesulfonate as a degradation product of dimethylsulfide produced by marine algae [41], and sulfoquinovose in the plant sulfolipid [42] (Table 2). Aeruginosin B, a phenazine derivative synthesized by *P. aeruginosa*, is the only known natural aromatic sulfonate [43]. The sulfur in aerobic soils is almost entirely found in organically bound form (Fig. 2), and has been characterized on the basis of chemical reactivity as hydriodic acid-reducible sulfur (sulfate ester sulfur), Raney-nickel reducible sulfur (amino acid sulfur) and residual organically bound sulfur (sulfonate sulfur). Whereas this last assignment appears somewhat generous, it has been confirmed by very recent studies of sulfur composition in marine sediments, and in soils. Using X-ray absorption near edge structure spectroscopy, Vairavamurthy and co-workers have shown that up to 37% of the sulfur in several sediment and humic substance samples was indeed sulfonate sulfur [44], and that sulfonates made up 20–40% of the organic sulfur in marine sediments down to at least 50 cm depth [45]. Low molecular mass sulfonates have also been found in micromolar concentrations in the porewater of marine microbial mats [46]. Sulfonates are also present in all soil strata, including the humus layer. They may be derived from biogenic sulfonates such as plant sulfonolipid, or they may be oxidation products of cysteine, as has been suggested for the sulfonates present in humic substances carried in river water [47]. It has also been suggested that sulfonates may arise in sediments by a chemical process involving the addition of sulfide to carbon-carbon double bonds [48]. Since sulfide is omnipresent in soils and sediments, due to the action of sulfate-reducing bacteria, this theory appears attractive. In situ labelling studies using radiolabelled sulfate in forest soils have shown not only that sulfate is rapidly incorporated into the sulfonate fraction, but that there is a significant flux of sulfur through this sulfonate pool, and that it ultimately reappears as sulfate [36,49,50]. Humus chemistry is complex, due to the enormous structural diversity of the substrate [51], but it seems clear that both sulfonate synthesis and desulfonation reactions take place in humus at considerable rates, and that much of this activity is microbially mediated.

Sulfur present in humus will ultimately make its way into sulfur-containing compounds in oil and coal. Research into specific desulfurization of such compounds, without degrading the backbone carbon structure, makes up an important objective of many research groups [52]. In the past 20 years, success in reducing the sulfur content of oil, primarily by hydrodesulfurization, has led to a dramatic decrease in SO_2 pollution in the industrialized world. An unexpected result of this success is the emergence of the phenomenon of sulfur limitation for crops, as the plants can no longer absorb the sulfur they require from the atmosphere, but are forced to rely on the lower levels of inorganic sulfate provided by the soil.

3.2. Production, role and fate of sulfonated xenobiotics

Xenobiotic sulfonates are used in a wide variety of applications, as diverse as dyestuffs and brighteners, detergents, cement additives, and industrial chemical intermediates. Many of these compounds, and intermediates from their synthesis, are released into the environment and can be detected in rivers [53–56] or in sewage sludge-amended soils [57,58]. Most applications make use of the amphiphilic nature of aromatic sulfonates, with a highly charged sulfonate group ($pK_a < 0$) attached to a lipophilic aromatic ring. The most widespread aromatic sulfonates are probably the linear alkylbenzenesulfonate surfactants (LAS), with an annual world production in 1995 of 2.8 million tons, predicted to reach 4 million tons by 2005 [59]. Although LAS are not toxic to higher organisms [60], they may have toxic effects on algae and invertebrates at the levels found in polluted waters [61–64]. They accumulate in some natural compartments, particularly in sediments and other anaerobic environments [65–67], but under aerated conditions are generally subject to ready biodegradation, and are quickly mineralized.

3.3. Naturally occurring sulfate esters

Unlike aryl sulfonates, aromatic sulfate esters are not common xenobiotic compounds. The widespread occurrence of bacterial arylsulfatases therefore provokes the question of the identity of the natural substrates of these enzymes. In part, the problem is a misleading one. Arylsulfatases have often been defined as such because they are capable of hydrolyzing standard aromatic sulfate substrates such as 4-nitrophenyl sulfate or nitrocatechol sulfate [68], although their natural substrates are aliphatic or carbohydrate sulfates. Nonetheless, aromatic sulfates are certainly found in nature. In mammals sulfation is a key step in the detoxification and excretion of aromatic xenobiotics, since the sulfate conjugates are generally more water-soluble and less reactive than the parent compounds. Sulfation may occur either directly on a hydroxyl group (e.g. of dietary catechols), or after initial hydroxylation by cytochromes P450 [69], and the conjugates are subsequently excreted in the urine or in the bile. It should be noted that although this mechanism is geared towards

detoxification, in occasional cases it may also lead to bioactivation of e.g. aromatic amines or benzylic alcohols to carcinogenic derivatives [70]. Bacteria also utilize this strategy for xenobiotic detoxification [71], though this does not appear to be widespread.

Sulfation of tyrosine residues is a common post-translational modification that occurs in the eukaryotic *trans*-Golgi network [72–74], and up to 1% of the tyrosines in a cell may be sulfated [75]. In mammals, sulfation and desulfation reactions regulate the concentrations of biologically active molecules such as estrogens, which are transported around the body as the sulfate esters and hydrolyzed in the target tissue to the active steroid [70]. Sulfate esters are therefore common, biologically active molecules to which bacteria are commonly exposed.

As described above, a large proportion of the sulfur in various soil and sediment environments is present as sulfate esters. These may arise in humus itself, through chemical sulfation of lignin-derived phenols, or they may be biogenic in nature. In addition to the aromatic sulfates described above, carbohydrate sulfates contribute to natural deposition of sulfate esters. Glycosaminoglycans (heparin and heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate) are highly *O*-sulfated and *N*-sulfated, and are important components of connective tissue, playing roles in modulation of many extracellular processes [76]. In vivo, these polymers are subject to lysosomal hydrolysis [77] (most of the mammalian sulfatases characterized so far are specific for carbohydrate sulfates [78]). A variety of glycosaminoglycan lyases have also been characterized from bacterial sources [76]. These are extracellular enzymes that cleave the glycosaminoglycan backbone to yield di- to hexasaccharides. Most of the bacteria that catalyze this reaction have been isolated from soil, suggesting that soil environments are rich in glycosaminoglycan products, presumably of animal origin.

Alkyl sulfate surfactants in detergent formulations have now been largely displaced from the market by the alkylbenzenesulfonates described above, though alkyl sulfates are still in use in special laundry applications, and in products such as toothpastes, antacids or car-cleaning shampoos [79], and are therefore released to the environment. It has long been recognized that both primary and secondary alkyl sulfate surfactants are rapidly degraded by environmental bacteria [60], and so they are not present in high concentrations in the environment. A number of naturally occurring alkyl sulfate esters are also known, ranging from methyl sulfate to long-chain, partially chlorinated aliphatic sulfates derived from algae (reviewed in [35]). The ability of bacteria from pristine sites to degrade xenobiotic alkylsulfates is believed to derive from their previous exposure to these natural compounds [80], since alkylsulfatases are found even in sites which have not been exposed to surfactant pollution [80,81].

From the above, it is clear that a variety of natural and xenobiotic sulfonates and sulfate esters are to be found in the environment. In principle, bacteria can utilize these compounds in at least two ways. They can degrade the carbon skeleton to provide carbon and energy for growth, in which case the sulfur atom is released in excess of growth requirements for sulfur. In this case, the processes concerned are regulated, as far as they have been investigated, primarily by substrate induction of the corresponding genes, and by catabolite repression. Alternatively, sulfonates and sulfate esters can be desulfurized to provide sulfur for growth, a process which is controlled by the sulfur supply to the cell (i.e. derepression in the absence of preferred sulfur sources such as sulfate). Both carbon-cycle and sulfur-cycle regulation have been observed for metabolism of organosulfates and organosulfonates in laboratory studies with pure bacterial cultures. In the following sections, the emphasis will be on metabolism of organosulfur compounds as part of the sulfur cycle in bacteria, concentrating on aerobic systems.

4. Sulfatases – from bacteria to humans

Sulfate esters provide a readily available source of sulfur for bacteria in soil and enteric environments, and microorganisms have responded with the synthesis of a battery of sulfatase enzymes. Many of these appear to be related, and their activity is dependent on a formylglycine residue within the active site. Although several bacterial arylsulfatases are well characterized, the structure of the alkylsulfatase enzymes and the genetic details of their expression are still almost completely unknown, and elucidation of these problems will provide interesting challenges in the future.

Sulfatases (EC 3.1.6.-) have been isolated from bacteria [35], fungi [9,31], algae [34,82,83], sea urchins [84–86], and higher eukaryotic organisms [78], but there is only scanty evidence for their occurrence in plants [87–89]. Because arylsulfatase activity is widely distributed in soils, it is often used as a test of soil quality, along with e.g. urease, amylase and alkaline phosphatase (e.g. [87,90–92]). At first sight, there appears to be a contradiction in the simultaneous presence of high levels of sulfate esters and sulfatases in soils, but this can be rationalized if one considers that much of the sulfate ester sulfur may be protected from sulfatase attack by soil structure [93–95]. In humans most sulfatases are located in the acidic environment of the lysosomes, and are involved in the desulfation of glycosaminoglycans such as heparan sulfate, chondroitin sulfate and dermatan sulfate [77,78]. Other mammalian sulfatases are located in the microsome and in the endoplasmic reticulum/Golgi apparatus [78]. As already noted, although these enzymes are known as arylsulfatases, their natural substrates are predominantly carbohydrate sulfates, and not aromatic sulfate esters. Genetic deficiencies in most of the individual sulfatase genes are associated with specific human diseases [78,96]. In addi-

tion, there is a rare lysosomal storage disease in humans called multiple sulfatase deficiency (MSD), in which all the sulfatases are defective. This syndrome is caused by the loss of the post-translational modification system that generates the essential formylglycine in the active site of all the sulfatases [97].

Bacterial arylsulfatases and alkylsulfatases were last reviewed in detail in 1982 [35]. Since that time there has been a considerable increase in our understanding of the genetics of arylsulfatases, and much progress has been made in understanding the mechanism of sulfatase action, both in eukaryotes and in microorganisms.

4.1. Bacterial arylsulfatases

Bacterial arylsulfatases have been identified and studied in a variety of species, including the enterobacteria (*Klebsiella* [98–100], *Salmonella* [101,102], *Enterobacter* [103,104], *Proteus* [105,106], *Serratia* [107]), pseudomonads (*Pseudomonas* [11,108,109], *Comamonas* [110]), mycobacteria, and cyanobacteria [111]. In recent years, however, genetic and biochemical studies have concentrated primarily on the arylsulfatase enzymes from *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, at least in part because these enzymes are closely related to the human arylsulfatases. Earlier work has been comprehensively reviewed [35], and is therefore only summarized briefly here.

Two main conclusions can be drawn from the early biochemical studies: (i) most of the strains studied contained multiple arylsulfatase isozymes, and (ii) the arylsulfatase enzymes can be divided into two groups according to pH optimum, with one group showing optimal activity at pH values 6.5–7.1, and the other group at higher pH values of 8.3–9.0. Thus, *Proteus rettgeri* was reported to contain nine arylsulfatase isozymes, with pH optima at 6.7 and 8.3 [105,112], *Serratia marcescens* contains multiple isozymes with a broad pH optimum of 6.8–8.2 [107], *P. aeruginosa* has two isozymes with pH optima 9.0 and 8.4 [109,113], and *Salmonella typhimurium* has five enzymes, all with optimum pH 6.7 [102]. It should be noted, however, that multiple arylsulfatase bands have also been reported in two species (*P. aeruginosa* and *K. pneumoniae*) where later work revealed only one functional arylsulfatase gene. The earlier observations of multiple isozymes may therefore be in part artefactual. Some of the earliest work on arylsulfatase was done with *Enterobacter (Aerobacter) aerogenes* (ATCC 9621). This strain has since been reclassified as *K. pneumoniae*, and so the data obtained [103,104,114,115] must be compared with later studies with the latter species.

Arylsulfatases are also commonly found in multiple forms in the mycobacteria, but there has been no attempt to characterize the enzymes in detail. However, arylsulfatase activity patterns have been used as a means of identifying and distinguishing different *Mycobacterium* species [116,117], and the presence of arylsulfatase is routinely used as a phenotypic test for the genus *Mycobacterium* [118].

4.1.1. The arylsulfatase enzymes of P. aeruginosa and K. pneumoniae

The bacterial arylsulfatases that have been studied most in the last decade are the enzymes from *K. pneumoniae* and *P. aeruginosa*. These two proteins are relatively similar at the protein sequence level (37% amino acid identity, see Fig. 3), and in size (60 kDa for the *Pseudomonas* enzyme [11], and 62 kDa for the mature *Klebsiella* sulfatase [119]. However, there are also significant differences, both at the protein and at the gene level. The *Klebsiella* sulfatase is a periplasmic enzyme, and analysis of the gene sequence reveals a typical signal peptide, whereas the *Pseudomonas* enzyme is an intracellular protein, since the N-terminal amino acid sequence corresponded with the 5′-end of the gene, and attempts to detect the *Pseudomonas* enzyme in periplasmic shock fluids were unsuccessful. The pH optimum for the *Klebsiella* enzyme is 7.5 [120], whereas the *Pseudomonas* sulfatase shows highest activity at a more alkaline pH value of 8.9 [11]. Two arylsulfatase isozymes were originally reported in *P. aeruginosa* [109,113], which differed in their isoelectric points and pH optima. Unfortunately these data were obtained with an incompletely characterized strain, and in more recent work, using the standard strain PAO1 [121], only one isozyme was detected by native isoelectric focussing, and in-gel activity staining [11]. In addition, deletion of the arylsulfatase gene (*atsA*) in this strain led to complete loss of arylsulfatase activity. The previously reported isozymes may therefore have been either experimental artifacts or degradation products, as has been suggested for additional sulfatase bands that appeared during purification of the *Klebsiella* enzyme [35]. However, it should be noted that two further putative sulfatase genes have been detected during sequencing of the *P. aeruginosa* genome [122] (see below), and it is possible that these genes are cryptic in strain PAO1 but were expressed in the strain studied previously.

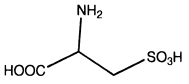
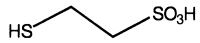
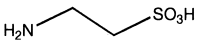
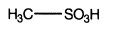
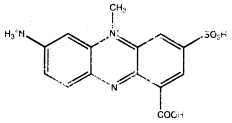
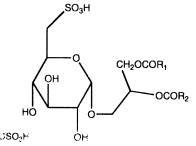
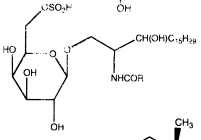
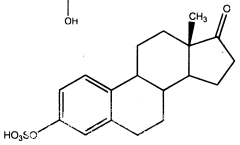
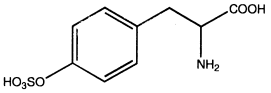
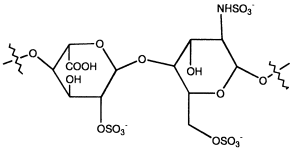
In addition to the enzymes described above (Fig. 3), a further sulfatase has recently been characterized from the marine bacterium *Alteromonas carrageenovora* [83]. The *Alteromonas* enzyme is considerably smaller (36 kDa) than the other bacterial sulfatases, and does not display sequence similarity to any of the sulfatases above. It shows a broad optimum in its activity at pH 8.5. The enzyme appears to be located in the periplasm, and is probably involved in desulfation of sulfated polysaccharides [83]. Although expression of the *Alteromonas* arylsulfatase is not repressed by sulfate [83], the protein is low in cysteine (only one residue), a typical characteristic of sulfate-regulated enzymes (cf. the *Klebsiella* arylsulfatase (zero cysteines) and the *Pseudomonas* arylsulfatase (zero cysteines)).

4.1.2. Post-translational modification of sulfatases

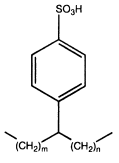
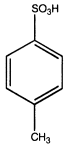
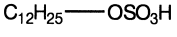
All the arylsulfatases characterized to date (Fig. 3), ex-

Table 2
Natural and synthetic sulfonates and sulfate esters

Natural sulfates and sulfonates

Structure	Compound	Source
	cysteate	Wool
	coenzyme M	methanogenic archaea
	taurine	mammals
	methanesulfonate	atmospheric photoxidation product of dimethylsulfide
	aeruginosin B	*Pseudomonas aeruginosa*
	sulfoquinovose	plant sulfonolipid in photosynthetic membranes
	cerebroside sulfate	human nerve tissue
	estrone sulfate	humans
	tyrosine sulfate	eukaryotic proteins
	heparin	a representative glycosaminoglycan

Xenobiotic sulfates and sulfonates

		
Linear alkylbenzene-sulfonate (LAS) (surfactant)	toluenesulfonate (hydrotropic agent)	dodecyl sulfate (surfactant)

cept for the *Alteromonas* enzyme, contain a conserved amino acid sequence motif in the active site, which is required for enzyme activity. This sulfatase motif (C/S-X-P-X-R-X_4-TG) is conserved in both prokaryotic and eukaryotic sulfatases, and in all cases examined it directs a post-translational modification of the initial cysteine or serine in the sequence to a 2-oxoalanine (formylglycine; FGly) residue. The presence of this FGly residue has been demonstrated

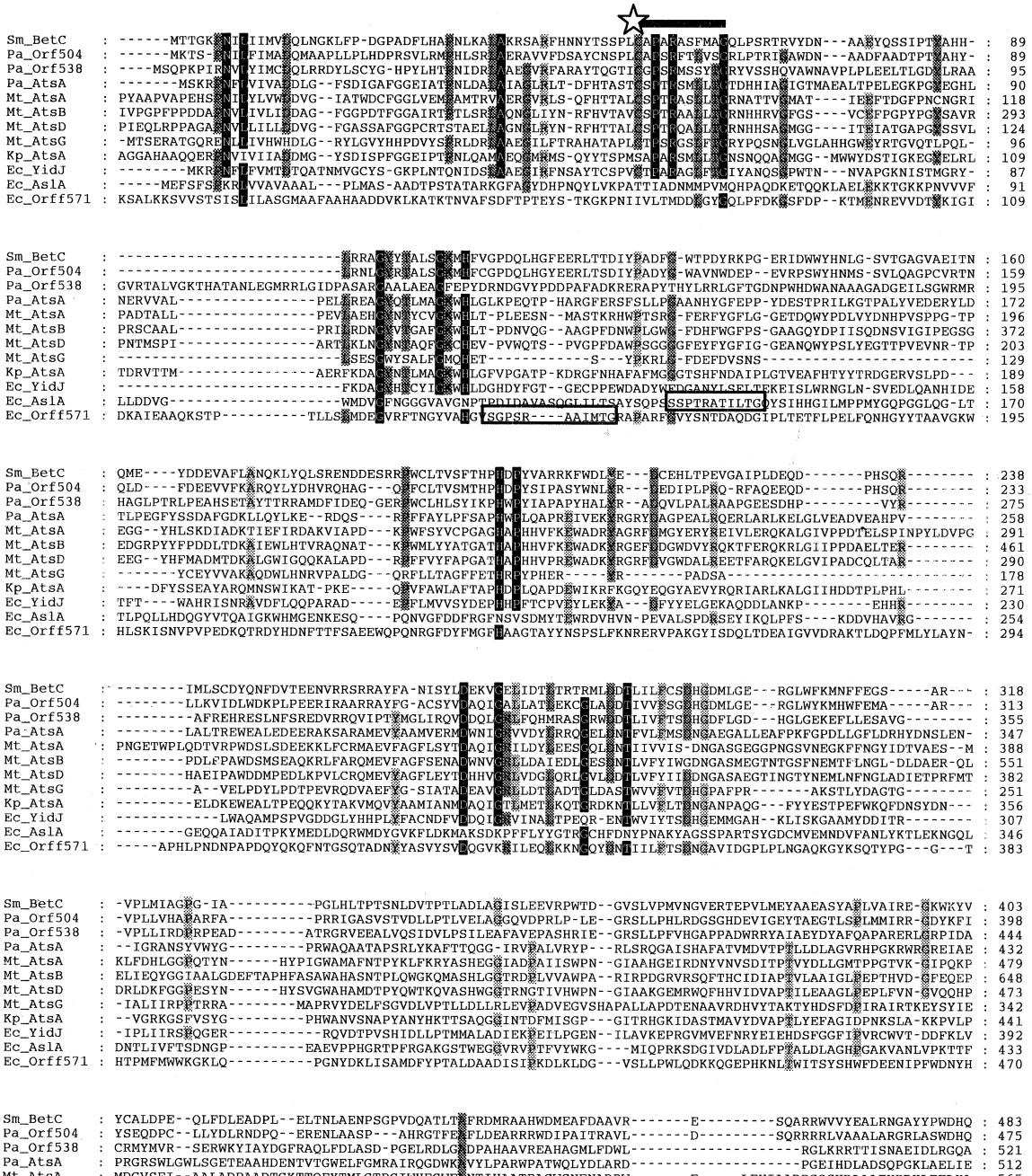


Fig. 3. Sequence comparison of bacterial arylsulfatases and related proteins. The proteins shown are: *S. meliloti* BetC (512 aa), *P. aeruginosa* Orf504 (504 aa), Orf538 (538 aa) and AtsA (536 aa), *M. tuberculosis* AtsA (787 aa), AtsB (970 aa), AtsD (787 aa) and AtsG (465 aa), *K. pneumoniae* AtsA (464 aa), *E. coli* YidJ (497 aa), AslA (551 aa) and Orf f571 (571 aa). The alignment was done with CLUSTALW, and identical residues are indicated by shading. The cysteine/serine that is modified to formylglycine is indicated with a star, and the consensus modification sequence motif (PROSITE Sulfatase box 1) is overlined. In the AslA and Orf f571 proteins, this consensus sequence does not align well with the other sequences, and is boxed separately.

in the human lysosomal arylsulfatases A [123,124] and B [125]. A lack of the ability to carry out the Cys→FGly modification is the cause of the lysosomal storage disease MSD, in which the activities of all the lysosomal sulfatases are severely reduced [97]. The FGly residue is also found in the sulfatase of the alga *Volvox carteri* [126], and in the two bacterial sulfatases from *P. aeruginosa* [13] and *K. pneumoniae* [98]. In *P. aeruginosa* it is formed by mod-

ification of a cysteine residue, as in the eukaryotic sulfatases, whereas in *K. pneumoniae* it arises by modification of a serine residue. Modification of the bacterial sulfatases was demonstrated by reduction of the aldehyde group of the purified enzymes with [^{3}H]sodium borohydride, tryptic digestion, and radiosequencing of the corresponding peptide. This revealed that cysteine-51 of the *Pseudomonas* arylsulfatase and serine-72 of the *Klebsiella* enzyme had been modified, and the nature of the modification was confirmed by MALDI-MS of the peptide in its native state, and after treatment with nitroaniline, which reacts with aldehyde groups [13]. A striking difference between the two enzymes was that the *Klebsiella* sulfatase was only circa 40% modified [98,119], whereas the *Pseudomonas* enzyme was 100% converted to the FGly form [13], probably reflecting a difference in the efficiency of the cysteine and serine modification processes.

X-ray structural analysis of the human arylsulfatases A and B [127,128] has shown that the FGly in these proteins is present in the active site of the enzyme, as its hydrate or sulfate ester hemiacetal, at the base of a positively charged pocket. This hydrate is thought to play an essential role in the putative catalytic mechanism outlined in Fig. 4. Replacement of the cysteine in the active site of the human sulfatases by serine led to a complete loss of enzyme activity [125]. It is therefore particularly interesting that the serine residue of the *Klebsiella* sulfatase can be modified to FGly. The initial study showed that this was carried out with arylsulfatase isolated from *K. pneumoniae* [98], but the enzyme was also active when expressed in *E. coli* [99,119]. The *Pseudomonas* enzyme was also active when expressed in *E. coli*, but all activity was lost when the cysteine-51 residue was replaced by a serine or an alanine [13]. Identical results were obtained when the *Pseudomonas* sulfatase and its C51S and C51A mutant forms were transformed into a Δ*atsA* strain of *P. aeruginosa* or into *K. pneumoniae* [13]. These data suggest that two separate systems exist for modification of cysteine or serine residues within the conserved sulfatase motif, and that the Ser-specific modifying system of *E. coli* is incapable of recognizing the serine form of the *Pseudomonas* sulfatase.

The proposed classification of sulfatase-modifying enzymes into Cys-specific and Ser-specific systems has mechanistic implications (Fig. 5). Conversion of a serine residue to FGly requires merely an oxidation, whereas the modification of cysteine to FGly proceeds formally via oxidation to the thio-aldehyde, followed by a hydrolytic step. The activation enzymes for serine and cysteine have not yet been identified in any organism, but they are therefore likely to be oxidoreductases. Schirmer and Kolter [129] have compared this reaction with the oxidative conversion of aspartate to oxaloacetate via iminoaspartate [130], but this has not yet been tested experimentally. These authors postulate an oxygen-dependent reoxidation of the cofactor. However, there must be other mechanisms, since the *Pseudomonas* arylsulfatase can be overexpressed in active form in *E. coli* cells grown under anaerobic conditions [131]. As mentioned above, the Cys-type *Pseudomonas* sulfatase is an intracellular enzyme, whereas the Ser-type sulfatase of *Klebsiella* is periplasmically located. This correlation between sulfatase type and putative cellular location has also been found in all other hypothetical sulfatases that have been identified through genome analysis (see below) This leads to the interesting possibility of a spatial separation between the two modifying systems, i.e. that the Cys-modifying system is located in the cytoplasm, whereas the Ser-modifying enzyme either is located in the periplasm or is obligatorily linked to transport through the membrane. An analogous situation to the latter is found for human sulfatases, for which the modification process occurs at a late stage of protein translocation into the endoplasmic reticulum [125].

Searching the databases for the consensus sequence for the arylsulfatase consensus motif led to the discovery of several other enzymes that contain this motif and catalyze related reactions. Thus, the alkaline phosphatase of *P. aeruginosa* (accession number AF047381) and the phosphonate monoester hydrolase of *Burkholderia caryophylli* [133] also contain the modification sequence, though it has not yet been shown that either of these is indeed modified in the active site. In addition, if the essential core sequence C/S-X-P-X-R [119] is combined in a search with mismatched versions of the auxiliary element AALLTGR, then further proteins are found that almost match the consensus [134]. It will be interesting to determine in future whether any of these indeed contain FGly, and if this modification therefore plays a wider role in cellular metabolism.

4.1.3. Arylsulfatase genes in P. aeruginosa and K. pneumoniae

An obvious candidate for the Ser-modification enzyme is found immediately when the genetic structure of the arylsulfatase gene in *K. pneumoniae* is examined. This is the $atsB_{Kp}$ gene product, which is encoded in a bicistronic operon with the $atsA_{Kp}$ gene, encoding the arylsulfatase (Fig. 6) [99,119] (for clarity's sake, a subscript is added to the *ats* genes here, *Kp* for *K. pneumoniae*, and *Pa* for *P. aeruginosa*). The $atsB_{Kp}$ gene product is required for synthesis of active arylsulfatase, and was originally proposed to be a transcriptional activator for the *atsBA* operon [135]. However, the 45-kDa AtsB protein does not show any of the motifs expected for a transcriptional regulator (e.g. helix-turn-helix DNA binding regions), and expression of the $atsA_{Kp}$ gene behind the strong *lac* promoter in *E. coli* in the absence of $atsB_{Kp}$ yielded only inactive arylsulfatase [119]. When this inactive arylsulfatase was purified, it was found that the serine at position 72 was completely unmodified. However, co-expression of the $atsA_{Kp}$ and $atsB_{Kp}$ genes on separate plasmids led to synthesis of an active sulfatase, in which the arylsulfatase *ser*-72 was 50% modified to FGly [119]. The AtsB protein

contains 12 cysteine residues in three clusters whose arrangement (cluster 1: C-X_3-C-X_2-C; cluster 2: C-X_5-C-X_{14}-C; cluster 3: C-X_2-C-X_5-C-X_3-C-X_{18}-C) is reminiscent of [Fe-S] iron sulfur centers [129]. This structure is consistent with the putative function of the modification enzyme as an oxidoreductase. The deduced protein does not carry a signal peptide, and appears to be a soluble, cytoplasmic protein. Since the *Klebsiella* arylsulfatase is a periplasmic enzyme, this implies that modification of the serine-72 residue occurs prior to translocation into the periplasm. An arylsulfatase-negative mutant of *K. pneumoniae* could be complemented with the $atsBA_{Kp}$ genes, demonstrating that this strain contains only one sulfatase isozyme that was expressed under the conditions used [99].

In *P. aeruginosa* the *ats* genetic organization is somewhat different. Since the *Pseudomonas* arylsulfatase is a cytoplasmic enzyme, an uptake system is required to transport the charged sulfate esters into the cell. The genes encoding such an uptake system are indeed found upstream of the $atsA_{Pa}$ gene (Fig. 6) ([136], accession number Z48540). Similarity searches revealed that the $atsBC_{Pa}$ genes are similar to the permease and ATP binding components of ABC-type transporters, respectively. Transporters of this type are normally composed of two permease components within the cell membrane, each of which is made up of six transmembrane helices, and two ATP binding subunits which are located peripherally to the cytoplasmic side of the cell membrane [137,138]. In addition, a high-affinity substrate binding protein located in the periplasm is responsible for delivering the specific substrate to the membrane component. The 58-kDa $atsB_{Pa}$ gene product is larger than most characterized permease components, and contains 12 putative transmembrane helices. It is therefore equivalent to a dimer of six-helix components, and presumably interacts with two molecules of the AtsC protein, which was identified as the ATP binding subunit by the presence of the conserved Walker motifs A and B [139,140]. The *atsB* and *atsC* genes overlap by four nucleotides. A fourth gene, $atsR_{Pa}$, lies divergent to the *atsBCA* operon, and encodes a periplasmic protein. The role of these proteins was confirmed by insertional inactivation of either the $atsR_{Pa}$ or $atsB_{Pa}$ genes with a gene cassette encoding gentamicin resistance (Gm) [136]. Both *atsR::Gm* and *atsB::Gm* mutant strains were unable to grow with aromatic sulfates, and whereas the *atsR::Gm* mutant still produced arylsulfatase, the polar effect of the $atsB_{Pa}$ insertion led to a loss of arylsulfatase activity as well. Complementation of the $atsB_{Pa}$ mutant with the $atsA_{Pa}$ gene under *lac* control led to restoration of arylsulfatase activity but not of growth with arylsulfates.

The $atsR_{Pa}$*::Gm* and $atsB_{Pa}$*::Gm* mutants are also defective in utilization of hexyl sulfate as a sulfur source, and the AtsRBC transport system therefore constitutes a general transporter for sulfate esters. It also plays a role in the transport of aromatic sulfonates, as discussed below. Interestingly, the *Pseudomonas* arylsulfatase does not hydrolyze alkylsulfate esters, so an alkylsulfatase gene must also be present elsewhere on the chromosome.

P. aeruginosa contains only one gene with significant similarity to the $atsB_{Kp}$ gene, *nirJ*, whose gene product shows 25% identity to $AtsB_{Kp}$ in the N-terminal domain. However, since the *nirJ* gene is part of an extensive ANR-regulated operon encoding the dissimilatory nitrite reductase [141], it seems unlikely that the NirJ protein is involved in sulfatase maturation. It is also interesting to note that *Synechocystis* contains an $atsB_{Kp}$ homologue (ORF *slr1507* [142]), but does not contain any genes encoding putative sulfatases. Together, these data suggest that (i) although the $atsB_{Kp}$ gene product is essential for Ser-type sulfatase maturation in enteric bacteria [119], other species have developed alternative strategies to carry out this modification, and (ii) there may be other proteins that require the FGly modification for activity.

4.1.4. Putative sulfatase genes in bacterial genome sequences

In recent decades there has been no systematic search for bacterial species that possess arylsulfatase activity, and since earlier screens were done before it was recognized that expression of bacterial arylsulfatase genes is often repressed in the presence of sulfate [35], the value of these studies is somewhat limited. Since the start of the 'genomic age' it has become possible to complement biochemical searches by in silico screening of published bacterial genome sequences for open reading frames with sequence similarity to known sulfatase genes. The arylsulfatase gene sequences from *P. aeruginosa, K. pneumoniae* and *A. carrageenovora* were used as probes to search the non-redundant GenBank database, the completed bacterial genomes, and the *P. aeruginosa* partial genome sequence for arylsulfatase homologues using the BLAST algorithm [143].

The results are summarized in Figs. 3 and 6, and were somewhat surprising. Putative sulfatases were found in *E. coli*, *P. aeruginosa*, and *Mycobacterium tuberculosis*, i.e. in exactly those species where arylsulfatase enzymes have already been studied. In the 19 other complete genomes and 20 incomplete genome projects examined, only three species contained arylsulfatase homologues: *Bordetella pertussis* [144] and *Salmonella typhi* [144] each contained two putative Cys-type sulfatases, and *Yersinia pestis* [144] had two Cys-type and one Ser-type sulfatases. The sulfatase sequences in these three organisms were not examined further, as the genome sequences are not yet complete. *P. aeruginosa*, *M. tuberculosis* and *E. coli* were also found to contain multiple arylsulfatase genes [122,145,146]. Thus, *P. aeruginosa* contained three arylsulfatase copies, of which one is presumably choline sulfatase [147], a second is an arylsulfatase [11], and the third is similar to a published phosphonate monoester hydrolase [133]. *M. tuberculosis* contained four different sequences with similarity to arylsulfatases [146]. The best described

set of sulfatase genes is the three putative sulfatases found in *E. coli* [29], and is particularly interesting because this organism does not grow with sulfate esters as sulfur sources, and the putative sulfatase genes are all cryptic. All the putative sulfatase genes encoded proteins which contained the FGly modification sequence, carrying either a cysteine or a serine in the position to be modified. The putative Ser-type sulfatases also carried a putative signal sequence, for export to the periplasm, as noted by Schirmer and Kolter [129], whereas the Cys-type sulfatases appear to be cytoplasmic.

More information can be obtained from the genetic environment of the putative sulfatase genes (Fig. 6). The Ser-type sulfatases found in enteric bacteria (*aslA* and *orf f571* in *E. coli* and $atsA_{Kp}$ in *K. pneumoniae*) are all found in close proximity to a gene encoding the putative activator protein (*aslB, orf f390* and $atsB_{Kp}$, respectively). An activator of this sort was not found near the genes encoding the Cys-type sulfatases (indeed, there is no evidence that either *P. aeruginosa* or *M. tuberculosis* contain an $atsB_{Kp}$ homologue). The Cys-type sulfatases almost all occur in gene clusters which encode transport proteins of some sort, underlining the putative location of the Cys-type sulfatases within the cell, and perhaps providing some evidence about their function. Thus, the *atsRBC* genes of *P. aeruginosa* are thought to be involved in uptake of aliphatic and aromatic sulfate esters, and the genes preceding the choline sulfatase in this species may be connected with choline sulfate transport. The *E. coli yidJ* gene, encoding a putative Cys-type sulfatase (Fig. 6), is distal to a gene encoding a putative glucose transporter, implying a possible function in glucose-sulfate uptake.

The presence of multiple arylsulfatase genes in each genome where sulfatase genes were found makes it tempting to draw conclusions about the occurrence of arylsulfatase isozymes often reported earlier [35]. For example, if one of the three sulfatases present on the genome of *P. aeruginosa* were cryptic in one isolate, and expressed in another, this would explain the observed differences in the number of isozymes in different reports [11,109]. Several species of enteric bacteria, in particular, seem to contain several sulfatase genes. It will be interesting to learn whether these are all pseudogenes, or whether the encoded proteins are expressed under conditions that have not yet been discovered and catalyze the desulfation of substrates that have not yet been tested.

4.1.5. Regulation of bacterial arylsulfatase expression

With the exception of the arylsulfatase from *A. carrageenovora* [83], expression of all the bacterial arylsulfatases which have been studied biochemically is subject to repression during growth in the presence of inorganic sulfate, and derepression during growth with organosulfur sources such as methionine or alkanesulfonates [11,21,35]. This regulatory pattern strongly suggests that the role of these enzymes in nature is exclusively in the assimilation of sulfur for bacterial growth, and that the arylsulfatases are an important part of the bacterial sulfate starvation response.

However, arylsulfatase expression is repressed not only by sulfate, but also during growth with cysteine or with cysteine biosynthetic intermediates such as sulfite or sulfide. This pattern of regulation is reminiscent of the control of cysteine biosynthesis, which has been best characterized in enteric bacteria [5,17]. In these species, activation of the genes of the *cys* regulon is mediated by the CysB protein, together with *N*-acetylserine, which acts as a co-inducer, and with sulfide or thiosulfate, which act as anti-inducers. In *P. aeruginosa*, expression of *atsB::lacZ* and *atsR::lacZ* fusions was also found to require an active CysB protein [136]. Although the pathways of cysteine and methionine biosynthesis in *P. aeruginosa* and *E. coli* differ slightly [19,20,148] (Fig. 1), this demonstrates that the arylsulfatase gene cluster constitutes an extension of the *cys* regulon in the former species.

The question of how sulfate, sulfite and sulfide cause gene repression in *P. aeruginosa* is more complex. In principle, sulfate may exert its repressive effect either directly or after conversion to another metabolite, probably (but not certainly) in the cysteine biosynthetic pathway. In order to isolate the effects of different intermediates of the pathway, defined mutants were constructed in the *cysN* (sulfate activation) and *cysI* (sulfite reductase) genes of *P. aeruginosa*, and the ability of sulfate, sulfite or sulfide to repress arylsulfatase synthesis in these mutants was studied (Table 3). Inorganic sulfate did not repress sulfatase formation in a *cysN* mutant, but did so in a *cysI* strain, suggesting that either PAPS or sulfite was acting as a repressor. *cysN* and *cysI* mutants grown with sulfide also did not synthesize arylsulfatase, and since cysteine does not repress arylsulfatase completely in *P. aeruginosa* [20], it seems likely that sulfide is also a repressor. At least two independent compounds are hence active as repressors in vivo in this species. Alternatively, the active repressor of sulfatase synthesis is a separate, uncharacterized molecule that can be synthesized from either of these two precursors.

A similar study was carried out in *K. pneumoniae* by Adachi et al. [100], but with very different results. Here, too, a strategy was adopted that made use of mutants in cysteine biosynthesis, but the mutants were defined only physiologically, and not at a genetic level. Synthesis of arylsulfatase was repressed independently by intracellular sulfate and by cysteine, but not by sulfite, sulfide or thiosulfate directly. Unfortunately, the role of CysB in regulation of arylsulfatase expression in *K. pneumoniae* has not yet been examined.

Arylsulfatase synthesis in enteric bacteria is regulated not only by sulfur supply, but also by monoamine compounds such as tyramine, dopamine, or norepinephrine. In the presence of tyramine, sulfate-mediated repression of arylsulfatase synthesis is overridden, and arylsulfatase is expressed, together with other genes of the monoamine

(*moa*) regulon, including monoamine oxidase (*maoA*), tyramine oxidase (*tynA*) and several uncharacterized genes [135]. This effect has been shown to be mediated by the positive regulator protein MoaR, a 26-kDa protein which is related to response regulators of the two-component sensor-regulator family. Expression of *moaR* is subject to catabolite repression [149], and the expression of arylsulfatase as part of the *moa* regulon may therefore imply its involvement in central metabolism. It has indeed been proposed [135] that the monoamine regulon in enteric bacteria is primarily concerned with utilization of dopamine sulfate, since dopamine, like many other phenols and catechols, is transported in human systems as its less reactive sulfate ester. A similar derepression of arylsulfatase expression by monoamine compounds has been observed in several other enteric bacteria, including *S. typhimurium*, *S. marcescens*, and also *E. coli*, in which the cryptic *atsA* gene was found to be induced by tyramine, using an *atsA::lacZ* fusion as a reporter system [150]. Tyramine does not derepress arylsulfatase synthesis in *P. aeruginosa* [11].

A mutant of *K. pneumoniae* has also been reported in which arylsulfatase was expressed constitutively, regardless of the sulfur source supplied [100]. The wild-type phenotype was regained when the mutant was transformed with the $atsR_{Kp}$ gene [151]. The encoded protein showed significant similarity (89% identity) to the dihydrofolate reductase of *E. coli*. Expression of the *atsR* gene on a plasmid led to increased dihydrofolate reductase activity in the cells, and the *E. coli folA* gene was also able to complement the *atsR* mutation in *K. pneumoniae*. However, the significance of this link between arylsulfatase synthesis and C-1 metabolism remains unclear.

A further important regulatory aspect of arylsulfatase synthesis is how synthesis of the corresponding modification system(s) is controlled. Very little is yet known on this subject, but it is clear already that the modification systems are not regulated by sulfur supply, since in *E. coli* both the *Pseudomonas* and *Klebsiella* arylsulfatases are expressed in active form after growth in LB medium, if the *atsA* genes are expressed behind the *lac* or T7 promoters [13,119]. It seems possible that the modification systems are important in another role that has yet to be identified, and that their expression is therefore either constitutive or regulated by other factors.

4.2. Alkylsulfatases

Alkylsulfatases differ from arylsulfatases not only in their substrate range, but also from a mechanistic point of view. Whereas arylsulfatases cleave the O-S bond of their substrates (a mechanism which is consistent with (but not indicative of) the presence of a FGly hydrate in the active site (Fig. 4)), the long-chain alkylsulfatases break the C-O bond and do not form a covalent enzyme-sulfate intermediate (reviewed in [35]). It therefore seems unlikely that the long-chain alkylsulfatases share the FGly modification found in the arylsulfatases. By contrast, the short-chain alkylsulfatase synthesized by the coryneform strain B1a also catalyzes cleavage of the O-S bond [152], but no further data are yet available concerning its mechanism.

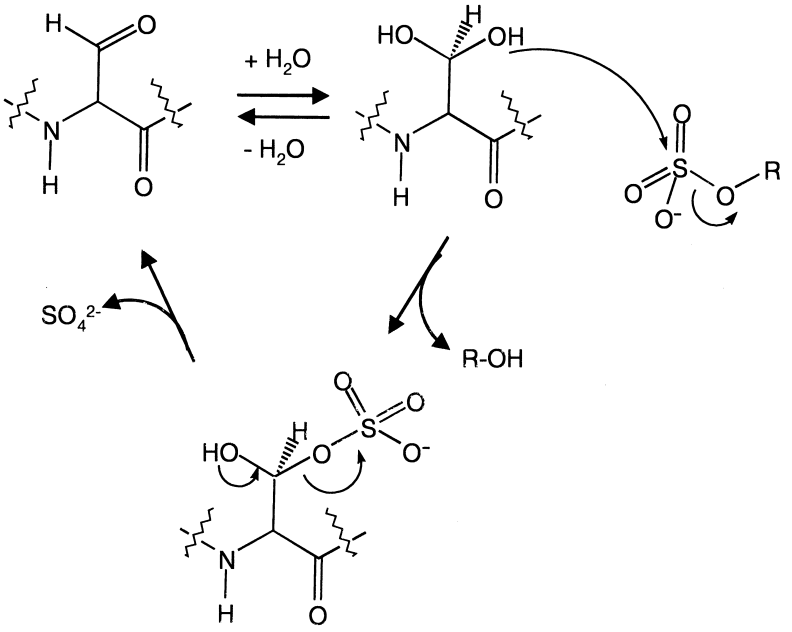


Fig. 4. Proposed mechanism of sulfate ester cleavage in arylsulfatases (adapted from [96]). The hydrate form of the active site formylglycine attacks the sulfate ester, breaking the S-O bond and forming a covalent enzyme-sulfate intermediate. This intermediate decomposes to regenerate the aldehyde form, which is then rehydrated in the active site by addition of a water molecule.

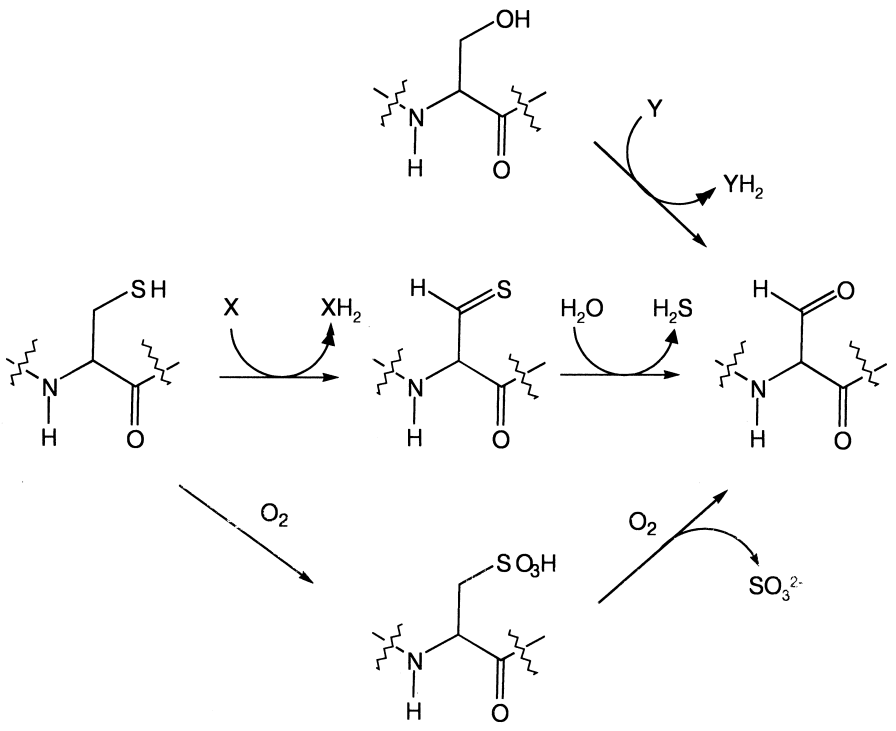


Fig. 5. Mechanisms for formylglycine formation in Cys-type and Ser-type arylsulfatases. Conversion of serine requires only an oxidative step, with unknown electron acceptor, whereas cysteine conversion requires an additional hydrolytic step. Alternatively, cysteine oxidation to cysteate and subsequent oxygenolytic cleavage would also lead to the desired aldehyde.

All of the alkylsulfatases described to date come from Gram-negative bacteria, though a *Bacillus cereus* strain is known that will grow with dodecyl sulfate [153]. Alkylsulfatases were included in an extensive review in 1982 [35], which detailed many of the earlier results obtained. Much of this work was carried out with the strain *Pseudomonas* $C_{12}B$, an isolate from a sewage outlet which synthesizes as many as five separate alkylsulfatases. Two of these are specific for primary sulfates, but differ in the pattern of their regulation, one being constitutively expressed, and the other inducible. The remaining three alkylsulfatases are specific for secondary sulfate esters; two of these are constitutive enzymes that specifically hydrolyze either D- or L-isomers, while the third secondary alkylsulfatase is an inducible enzyme. The two inducible enzymes are also repressed by primary alcohols (the products of the sulfatase reaction), and by tricarboxylic acid cycle intermediates, suggesting that catabolite repression plays a role in regulation of these enzymes.

This regulatory pattern immediately suggests that these enzymes are not part of the sulfur cycle, but are used to provide carbon and energy for bacterial growth. It should be noted, however, that most alkylsulfatase studies have been done with enzymes from bacterial strains that were isolated on the basis of their ability to mineralize sulfate esters completely (*Pseudomonas* $C_{12}B$, for instance, was isolated for its ability to grow with dodecylsulfate as carbon source [154]). In their earlier review, Dodgson et al. [35] listed only two alkylsulfatases whose expression was repressed by inorganic sulfate: the lithocholate sulfatase of *P. aeruginosa* [155] and cholinesulfatase of *Pseudomonas* V-A [156]. In both these studies it is notable that the sulfate ester was supplied solely as sulfur source, and carbon for growth was provided by benzoate or citrate, respectively. It seems likely that more sulfate-regulated bacterial alkylsulfatases still await discovery if suitable growth conditions are chosen.

Studies with the *Pseudomonas* $C_{12}B$ alkylsulfatases revealed that these were specific for medium- or long-chain sulfate esters, but had little or no activity with substrates with a chain length less than C_5. However, organisms have subsequently been isolated from soil, canal water and sewage which are able to grow with short-chain alkylsulfates as carbon source [157], and several of these have been examined in more detail. The sulfatase of the coryneform isolate B1A was active on C_3–C_7 primary alkylsulfates, but not on longer or shorter-chain homologues, nor on choline sulfate [152]. Methyl and ethyl sulfates acted as inhibitors of this enzyme (K_i values of 5.3 mM and 3.9 mM respectively), though these compounds were not themselves hydrolyzed by the enzyme. In two environmental isolates, *Agrobacterium* sp. strain M3C and *Hyphomicrobium* sp. strain MS223, methyl sulfate was not metabolized by a hydrolytic mechanism, but via an oxidative pathway [158,159]. Although initial studies had suggested that the *Hyphomicrobium* pathway might involve a hydrolytic enzyme, an elegant study using ^{13}C-labelled methyl sulfate demonstrated that in both species methylsulfate is

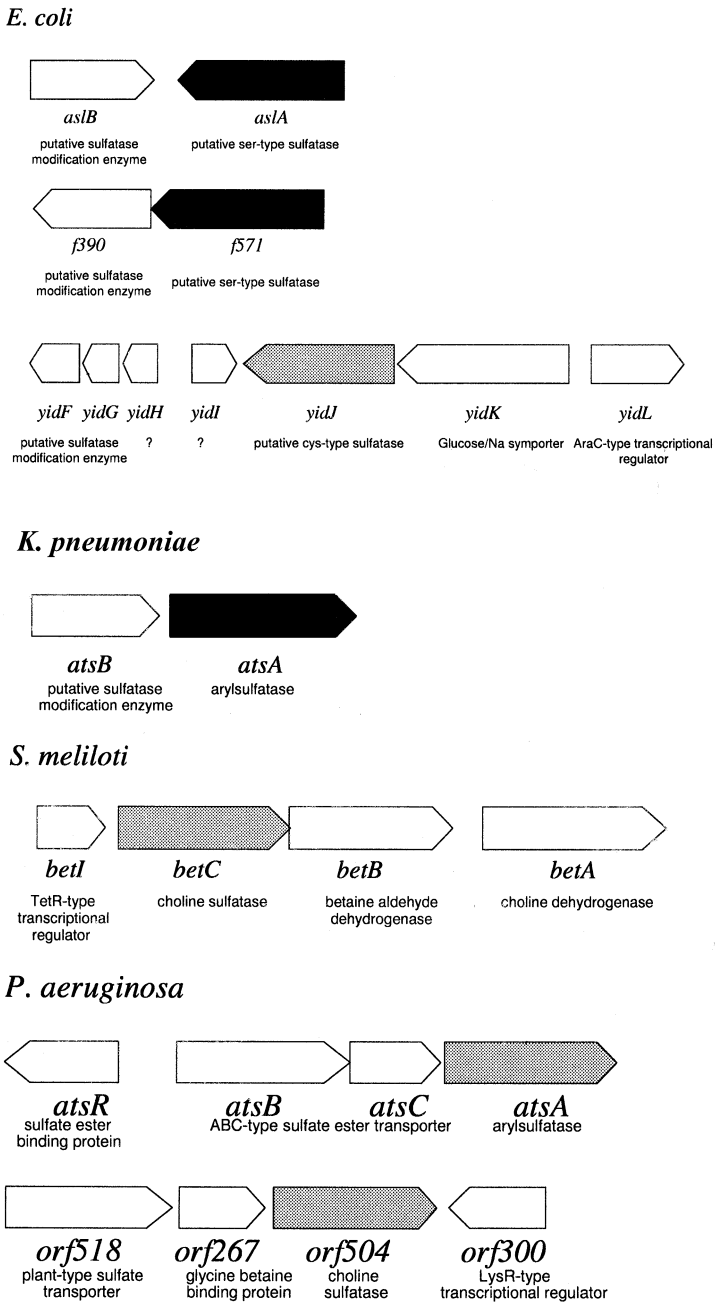


Fig. 6. Genetic structure of selected bacterial sulfatase gene clusters. Hypothetical function assignment of uncharacterized ORFs was done using BLAST [143]. The sequences are from: *E. coli* [129,145], *P. aeruginosa* [11,122,136], *K. pneumoniae* [99,119], *S. meliloti* [162]. Genes containing Ser-type sulfatases are shaded black, and those encoding Cys-type sulfatases are shaded gray.

converted directly to formaldehyde and sulfate, and that methanol is not an intermediate in this process [160]. A mechanism was proposed involving initial oxygenation to methanediol monosulfate by a monooxygenase, followed by spontaneous decay of the hemiacetal to yield formaldehyde and sulfate. Electron microscopic studies have revealed that whereas the long-chain-specific sulfatases of *Pseudomonas* sp. strain $C_{12}B$ were localized on the outer cell wall, the short-chain-specific enzymes in a coryneform species were intracellular [161].

From a genetic point of view, very little is known about alkylsulfate degradation, either in the carbon cycle or in the sulfur cycle. Gene sequences have been determined for a choline sulfatase (*betC*) from *Sinorhizobium meliloti* [162], for a bile acid sulfatase from *Comamonas testosteroni* [163,164], and for a putative dodecylsulfate sulfatase (*sdsA*) from the detergent-degrading strain *Pseudomonas* sp. ATCC 19151 [165,166]. The proteins encoded by the two latter genes appear to be related, and do not contain an FGly modification motif. The *betC* gene from *S. meli-*

loti, by contrast, belongs to the same family as the eukaryotic arylsulfatases and the *P. aeruginosa* arylsulfatase, and is a Cys-type sulfatase that appears to be located within the cell (no signal sequence was found) (Fig. 6).

The alkylsulfatase gene of *Pseudomonas* sp. ATCC 19151, *sdsA*, was identified by complementation of several mutants derived from a previously described SDS-utilizing strain. Two genes were identified that were required for SDS utilization, *sdsA* and *sdsB*. The putative SdsB protein is a member of the LysR family of transcriptional regulators, and is closely related to the regulatory protein required for arylsulfonate desulfonation in *P. putida*, described below. The regulatory role of the SdsB protein was confirmed by the fact that the SDS-negative phenotype of an *sdsB* mutant could be complemented by an *sdsA* gene expressed constitutively behind the T7 promoter. SdsB therefore seems to be required for expression of the *sdsA* gene, which encodes a 59-kDa protein. This protein was expressed in *E. coli*, but did not show detectable sulfatase activity. The lack of a signal peptide suggests that the encoded protein is intracellular, which contrasts with the observations that sulfatase activity in *Pseudomonas* sp. ATCC 11951 is periplasmically located, and it was suggested that the secretion sequence might be an atypical one, and not recognized in the heterologous host. Homologues of SdsA are found in *E. coli* (YjcS; 73 kDa, 32% amino acid identity to SdsA), and in *P. aeruginosa* (30% identity over the full length of the protein), and are related to lactamases from several organisms.

A homologue of SdsA (27% identity) has also been identified in *Comamonas (Pseudomonas) testosteroni*, where it catalyzes the hydrolysis of bile acid sulfates [163]. The organism was identified in a collection of strains for its ability to utilize lithocholic acid as a source of carbon and sulfur for growth, and the sulfatase was isolated and found to be a homodimer of 53-kDa subunits. Sulfatase activity was Mn^{2+}-dependent. Synthesis of the sulfatase was found to be completely repressed by 800 μM inorganic sulfate. Since the organism was grown with 2 mM lithocholic acid sulfate as carbon source, it is not clear why the excess sulfate released as a result of the differential cellular requirements for carbon and sulfur did not repress synthesis of the enzyme in vivo. One can speculate that the sulfatase is involved only in release of the sulfur, whereas the carbon-based degradation of the steroid skeleton was carried out by a separate system, leaving the sulfate ester bond intact. A second, biochemically similar sulfatase has recently been isolated from the same organism [167], but sequence data have not yet been reported.

A further type of alkylsulfatase has recently been reported in *S. meliloti* [162]. The BetC protein catalyzes the hydrolysis of choline sulfate to choline, and is part of a pathway that synthesizes the osmoprotectant glycine betaine. However, the organism can also utilize choline sulfate as a source of carbon, nitrogen and sulfur for growth. Expression of the sulfatase was induced by choline or choline sulfate, but not by sulfate limitation. This induction was slightly decreased under osmotic stress, and the sulfatase may therefore be involved in the metabolism of choline, rather than in osmoprotectant synthesis (choline sulfate itself showed only low osmoprotectant activity). Choline sulfatase has also been characterized from *P. aeruginosa* [147]. Its synthesis is tightly regulated in this species, since the level of enzyme produced depended on whether the substrate was being utilized by the cell as a source of carbon (22.8 nmol min^{-1} mg^{-1} protein), nitrogen (18.5 nmol min^{-1} mg^{-1}), or sulfur (4.1 nmol min^{-1} mg^{-1}). The product of the putative *P. aeruginosa* choline sulfatase gene (*orf504*, Fig. 6) [122] shows 45% identity to the *S. meliloti* BetC protein, and both contain the signature for a Cys-type sulfatase. Inhibitor studies carried out with the *P. aeruginosa* choline sulfatase [147] are consistent with a FGly residue constituting the active site as in arylsulfatases, but the presence of the modification has not yet been demonstrated.

4.3. Carbohydrate sulfatases

Most of the eukaryotic sulfatases are in fact carbohydrate sulfatases, acting on glycosaminoglycans as substrates [78]. Several bacterial sulfatases have also been characterized that belong to this group, including enzymes from *Proteus vulgaris, Flavobacterium heparinum* (now called *Cytophaga heparina*), and *Bacteroides thetaiotaomicron*. Glycosaminoglycan breakdown by these bacteria has been reviewed in detail [76], and involves the cleavage of the polymer by a lyase, usually extracellular, followed by desulfation of the resultant sulfated disaccharides and hydrolysis to the monosaccharide level by a glucuronidase. Bacteria that can carry out this process have been isolated primarily from soil, and degrade glycosaminoglycans as a source of carbon and energy for growth. No attempts appear to have been made to isolate bacteria that can utilize glycosaminoglycans solely as sulfur source for growth.

A further family of bacterial carbohydrate sulfatases has also recently been identified, which cleave the sulfate moiety from mucin. Mucin is particularly heavily sulfated in the mouth and in the colon, where bacteria are normally common, and the sulfation appears to help provide the mucin with a degree of protection from bacterial attack [168]. Strains of *B. thetaiotaomicron, P. aeruginosa* and *Burkholderia cepacia* were found to contain high levels of mucin sulfatase activity [169,170], a finding which is of importance in studies of cystic fibrosis, since the latter two species contribute significantly to the severity of this disease. Interestingly, an *E. coli* strain was also found to contain mucin sulfatase activity [170], and this may suggest a role for the uncharacterized sulfatase genes previously identified in the *E. coli* genome sequence [129].

The only putative bacterial carbohydrate sulfatase for which genetic data are available is the arylsulfatase of the marine Gram-negative aerobe *A. carrageenovora*. This enzyme has been proposed to play a role in degradation of sulfated fucans from brown algae [83], though these compounds were not directly tested as substrates for the enzyme. At the level of sequence similarity the *Alteromonas atsA* gene product is not related at all to the arylsulfatases described above, and also lacks the conserved sulfatase motifs required for generation of the active site formylglycine residue that it present in the latter proteins. However, sequence homology searches indicate that the *Alteromonas* sulfatase is part of a family of proteins that include the product of the *E. coli elaC* gene, and a number of hypothetical proteins from several other species. No function has yet been assigned to other members of this family, though the *elaC* gene may be involved in lanthanide resistance (G. Huisman, personal communication), but they share a zinc binding motif with the glyoxalase and β-lactamase families [171]. These genes have nonetheless been grouped together as the AtsA/ElaC family [171], and many have been annotated in GenBank as putative sulfatases. The dissimilarity of this protein family to most characterized sulfatases underlines the dangers of assigning function directly from protein sequence similarities without further biochemical analysis.

Since most of the bacterial carbohydrate sulfatases are involved in pathways involved in utilization of glycosaminoglycans as carbon sources, it is not surprising that their expression is induced by their substrates, and is subject to catabolite repression. Most studies of glycosaminoglycan degradation have concentrated on the lyase enzymes, and have paid little attention to the sulfatases. Thus, synthesis of the chondroitin sulfate lyases (chondroitinases) from *C. heparina* and *P. vulgaris* is induced by chondroitin sulfate or dermatan sulfate in the growth medium [76]. In *B. thetaiotaomicron* chondroitinase was induced only by the polymeric substrate, and not by its disaccharide breakdown products [172]. In this species all the enzymes in the degradation pathway (lyase, two sulfatases, and glucuronidase) are induced by chondroitin sulfate [172], and repressed during growth with glucose [173], suggesting that catabolite repression may play a role in regulating expression of this pathway. The specificity of the chondroitin sulfatases from *C. heparina* has been studied, with the aim of using these enzymes as tools in structural studies of chondroitin sulfate. Chondro-4-sulfatase was found to desulfate sugar residues both at the reducing end and at internal positions of an oligosaccharide, whereas chondro-6-sulfatase could only attack the end positions [174].

Degradation of heparin and heparan sulfate has been best studied in *C. heparina*. The breakdown pathway is initiated in this species by one of a family of three different heparinase enzymes that are located in the periplasm, and induced during growth with heparin. The resulting oligosaccharide sulfates are degraded further to the *N*- and *O*-sulfated monosaccharides. Desulfation is then carried out by a suite of monosaccharide sulfatases, several of which have been purified and characterized. These include a glyco-6-*O*-sulfatase [175], a glyco-3-*O*-sulfatase [176], both of which appear to be specific for monosaccharide residues, and a glyco-2-*O*-sulfatase which also accepts the terminal residue of oligosaccharides as substrate [177]. In addition, a sulfamidase has been purified from the same organism [178]. The glycosulfatases showed molecular masses of 58–64 kDa, very close to those reported for the bacterial arylsulfatases, and had optimum activity at pH 7.5–8.0 for the 6-*O*- and 3-*O*-sulfatases and pH 5.5–6.5 for the glyco-2-*O*-sulfatase. By contrast, the sulfamidase is somewhat larger in size (81.5 kDa), with a pH optimum of 7.0. No sequence data for these proteins are yet available at either the DNA or the protein level.

Evidence that glycosulfatases may be related to arylsulfatases comes from a study using *B. thetaiotaomicron*. A transposon mutant of this organism in the gene *chuR* was found to be unable to utilize either heparin or chondroitin sulfate as growth substrates [179]. This double phenotype was unexpected, since the two degradation pathways were thought to be independent of each other in this species. Biochemical characterization revealed that the mutant strain was defective in one of the two sulfatases involved in chondroitin sulfate degradation, chondroitin-6-sulfatase. Since the strain contains a second sulfatase, chondroitin-4-sulfatase, it was not clear why growth with chondroitin sulfate should have been disrupted, but as a chondroitin-4-sulfatase mutant was also unable to grow with chondroitin sulfate, a toxic effect due to accumulation of sulfated disaccharides was suggested. In addition, 2D-PAGE analysis of the *chuR* mutant showed reduced or zero synthesis of a number of proteins that were induced in the wild-type by chondroitin sulfate or hyaluronic acid (a related, non-sulfated polysaccharide) [180]. The initial conclusion was therefore that *chuR* encoded a regulatory gene that affected expression of chondroitin-6-sulfatase and an unidentified component of the heparin degradation pathway [179].

When the *chuR* gene was sequenced, it was found that the ChuR protein is 35% identical to the $AtsB_{Kp}$ protein of *K. pneumoniae*, which is required for expression of active *Klebsiella* sulfatase, and is thought to be involved in the modification of the active-site serine to a FGly (see above) [119,129]. It has therefore been proposed that the *chuR* gene product is responsible for a similar activation step of chondroitin-6-sulfatase, and of a sulfatase or sulfatases involved in heparin degradation [129]. If this is true, it would seem likely that the *B. thetaiotaomicron* sulfatases are Ser-type sulfatases, since $AtsB_{Kp}$ is involved in activation of a Ser-type sulfatase in *K. pneumoniae* [119]. Two arguments speak against this hypothesis. First, the *B. thetaiotaomicron* chondroitin sulfatases have been shown to be intracellular [181], and all the Ser-type sulfatases identified to date carry a signal sequence for export

to the periplasm. Secondly, the changes in protein expression observed by 2D-PAGE in the *chuR* mutant are inconsistent with a role of ChuR solely as a modifying protein, though such an effect could be caused by decreased stability of the target proteins if they remain unmodified (as has been observed for the C51A mutant of *P. aeruginosa* arylsulfatase [13]). At least one sulfatase in *B. thetaiotaomicron* (chondroitin-4-sulfatase) also remained unaffected by the *chuR* mutation.

The regulation of the heparin degradation pathway has also been well studied, primarily in *C. heparina*. The earlier part of this work has been reviewed by Dodgson et al. [35]. Heparin lyase is most strongly induced in the presence of heparin or heparan sulfate, although the disaccharide breakdown products are also good inducers [182]. However, induction was also seen in the absence of heparin when the cells were grown in a sulfate-free medium with methionine as sole sulfur source [183]. During continuous growth in low-sulfate medium with methionine as sulfur source, synthesis of heparinase or heparin sulfatase could be temporarily turned off by addition of limited amounts of sulfate [16]. The critical concentration causing repression was found to be between 10 and 70 μM, at which levels the sulfatase activity was not itself directly inhibited. Similar concentrations of sulfate have been shown to repress desulfurization of sulfonates and sulfate esters in *P. putida* [184]. In *C. heparina*, repression of both heparinase and sulfatase was seen when cysteine was added. As pointed out by Cerbelaud et al. [16], the immediate drop in enzyme activity after addition of sulfate implies either the presence of specific proteases, or a high endogenous turnover rate for the enzymes. Similar arguments have been invoked by Beil et al. [184] to explain the repression of aromatic sulfonatases by sulfate or cysteine observed in *P. putida* S-313 (see below).

Bacteria hence synthesize a range of sulfatases in order to use the sulfur available to them as sulfate esters, and in the cases that have been studied, the hydrolytic mechanism involves a novel active-site residue, FGly, rather than a serine as might be expected by comparison with phosphatases. Cleavage of sulfonates also follows novel pathways in the sulfur cycle, as will be seen below.

5. Novel oxygenases in the desulfonation of aliphatic sulfonates

The widespread occurrence of sulfonates in natural environments (Table 2) leads one to expect that a range of bacteria may have evolved the capacity to degrade them as either carbon or sulfur sources for growth, and this is indeed the case. Recent studies have shown that aerobic bacteria that use sulfonates as sulfur sources catalyze the desulfonation reaction using one of several unusual oxygenases, including α-ketoglutarate-dependent dioxygenases [12] and monooxygenases that make use of reduced flavin as a cosubstrate [14,132,185], and not as a bound prosthetic group.

Early biochemical work on sulfonate degradation concentrated on isolates that are able to use the carbon of compounds such as *n*-alkanesulfonates or taurine. *Pseudomonas* isolates that can degrade simple alkanesulfonates have been known for many years [186]. The initial desulfonation step in these strains is catalyzed by a monooxygenase, and yields the corresponding aldehydes. These isolates were able to grow with a range of straight-chain sulfonates (C_4–C_7 and C_8–C_{12} for the two strains studied), and presumably synthesize a single enzyme which accepts a range of substrates [187]. Oxygenolytic cleavage of the carbon-sulfur bond is also involved in degradation of the secondary alkanesulfonate sulfosuccinate, in *Pseudomonas* sp. B1. Monooxygenation at the α-position to the sulfonate yielded the unstable bisulfite adduct of oxaloacetate, which was proposed to decompose spontaneously to sulfite and oxaloacetate [188]. A similar reaction was observed in sulfonate-utilizing *Comamonas acidovorans* and *Rhodococcus* isolates [189], which desulfonated the branched-chain 2-propanesulfonate to acetone co-metabolically, although they were only able to grow with linear alkanesulfonates. This type of spontaneous decomposition of α-substituted sulfonates has been proposed to play a key role in many desulfonation steps involving oxygenative activation [190]. A similar type of reaction is also found in the methanesulfonate utilization pathway. Methanesulfonic acid is a major atmospheric decomposition product of dimethylsulfide, and can be utilized by several methylotrophic isolates as a source of carbon for growth [41,191]. The initial step in methanesulfonate mineralization in *Methylosulfomonas methylovora* [192] is catalyzed by a multicomponent methanesulfonic acid monooxygenase system (MSAMO) that hydroxylates methanesulfonic acid to the unstable intermediate hydroxymethanesulfonate [193–195]. This then decomposes to sulfite and formaldehyde, a common intermediate in methylotrophic metabolism [196]. MSAMO consists of a hydroxylase and an electron transport system (NADH reductase/ferredoxin); the latter transfers electrons from NADH via a [2Fe-2S]-Rieske center to the hydroxylase [194]. The hydroxylase consists of a two-component system (MsmAB) of the mononuclear iron type, which is most similar in sequence to dioxygenases containing Rieske centers [197]. MSAMO is therefore an atypical monooxygenase, since these usually lack the ferredoxin component, though a similar situation is known for two toluene monooxygenases [198,199] and is also seen in sulfate-regulated aromatic desulfonation in *P. putida* [185] (see below).

Taurine utilization as a carbon source, by contrast, can proceed by two pathways, neither of which involves oxygenation. In *Pseudomonas* [200,201] an initial step is required to activate the sulfonate bond by transamination to sulfoacetaldehyde, and this intermediate is then cleaved by sulfoacetaldehyde lyase to acetate and sulfite. Alterna-

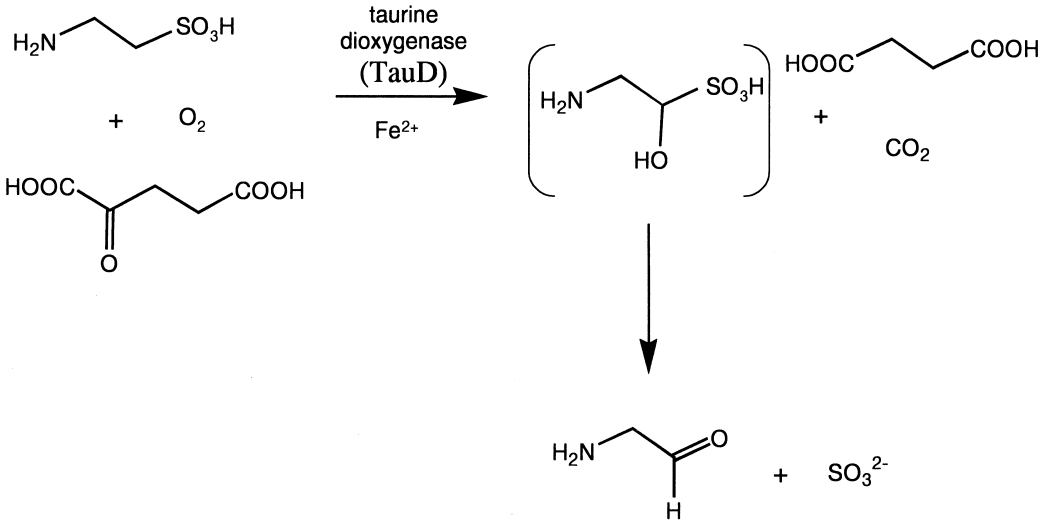


Fig. 7. Proposed mechanism for taurine desulfonation by the α-ketoglutarate-dependent taurine dioxygenase TauD [12]. Oxygenation of taurine leads to an unstable α-hydroxysulfonate intermediate, which decomposes to yield the product aminoacetaldehyde with release of sulfite.

tively, a taurine dehydrogenase may be involved, which catalyzes oxidative deamination to the sulfoacetaldehyde [202]. This compound is a key intermediate in the metabolism of natural sulfonates, as it is also involved in isethionate degradation in *Acinetobacter* and sulfoacetate utilization in *Comamonas* and *Aureobacterium* strains [204], though cell-free conversion of the sulfoacetate could not be observed in the latter isolates. The taurine degradation pathway via sulfoacetaldehyde, involving a taurine:pyruvate aminotransferase and a thiamine pyrophosphate-dependent sulfoacetaldehyde lyase is also active in several anaerobic species, including *Alcaligenes* sp. [205], *Clostridium pasteurianum* [206] and the novel isolate, strain GKNTAU.

Surprisingly, there seems to have been little work on the bacterial degradation of plant sulfonolipid. Since it is an important component of the photosynthetic membranes in all plants and algae, and most photosynthetic bacteria, it is probably the commonest sulfonate on earth, and plays an important role in the sulfur cycle [42]. Although the bleaching processes that take place during plant senescence involve chloroplast degradation, metabolism of the sulfolipid appears not to be catalyzed by the plant, and recycling of the sulfur is carried out purely by soil bacteria [208], but this process has not yet been investigated in detail.

The ability of bacteria to use aliphatic sulfonates as carbon sources is often limited to strains which have been specifically isolated using enrichment cultures that select for this phenotype. By contrast, natural sulfonates such as cysteate, isethionate or taurine can be used as a sulfur source for growth by a wide range of enteric bacteria, soil bacteria and yeasts [209–213], reviewed by Seitz and Leadbetter [214]. Of 100 random bacterial strains isolated nonselectively from freshwater, soil and freshwater sediments, 94 were able to grow with taurine as sulfur source, 96 with isethionate, and 87 with sulfoacetate [213]. By contrast, only about 10% of the strains were able to utilize the carbon moieties of these molecules for growth. Synthetic alkanesulfonates (including fluorinated sulfonates [215]) can also be used as sulfur sources by many species, and physiological experiments with *P. aeruginosa* [216] showed that desulfonation of several synthetic alkanesulfonates in the sulfur cycle appeared to follow the same pathway as in the carbon cycle, i.e. via monooxygenation and release of sulfite from a hydroxy-

Table 3
Synthesis of active arylsulfatase during growth of *P. aeruginosa* [20] or *K. pneumoniae* [100] with various inorganic sulfur-containing compounds

Strain	Mutation	Sulfur compound tested		
		sulfate	sulfite	sulfide
P. aeruginosa				
PAO1	wild-type	−	−	−
AC309	sulfate activation (*cysN*)	+	−	−
AX18	sulfite reductase (*cysI*)	−	−	−
K. pneumoniae				
W70	wild-type	−	−	−
K170	sulfate transport	−	+	+
K152	sulfite reductase	−	−	+

sulfonated intermediate. When the sulfur-regulated desulfurization process was examined at the molecular level, however, a different set of enzymes was found to those used to provide carbon for growth. Two main enzymatic desulfonation routes have been characterized, an α-ketoglutarate-dependent dioxygenase pathway that is active with taurine (Fig. 7), and an $FMNH_2$-dependent monooxygenase system that catalyzes the desulfonation of a range of aliphatic sulfonates (Fig. 9). Both of these systems have been found in several Gram-negative species, and they are examined in more detail below.

5.1. Taurine desulfurization; α-ketoglutarate-dependent dioxygenases

In *E. coli,* physiological experiments showed that taurine sulfur enters the cysteine biosynthesis pathway at the level of sulfite [217], but the detailed biochemistry of this reaction has only recently been elucidated. The α-ketoglutarate-dependent taurine dioxygenase of *E. coli* (TauD) was originally identified during screening for *E. coli* proteins whose synthesis was repressed in the presence of sulfate, but expressed during growth with alkanesulfonates [22] (see above). The corresponding gene, *tauD*, and two further genes in the same operon, *tauB* and *tauC*, were also identified as sulfate-repressed genes using a genetic screening strategy with a bank of λp*lac*Mu*9* insertion mutants [218]. From sequence comparisons, the TauD protein belongs to the family of α-ketoglutarate-dependent dioxygenases, and this has been confirmed by biochemical studies with the purified TauD protein [12]. The immediate product of taurine oxygenation by TauD is an unstable hydroxytaurine intermediate, which desulfonates spontaneously to yield aminoacetaldehyde and sulfite (Fig. 7). The product appears similar to that which would arise from a monooxygenase reaction. However, whereas monooxygenases transfer one oxygen atom of the oxygen molecule to an organic substrate and the second atom to water, α-ketoglutarate-dependent dioxygenases transfer both oxygen atoms to organic acceptor molecules. One oxygen atom is used to hydroxylate the reaction substrate itself, and the second is transferred to the cosubstrate, usually α-ketoglutarate, which is thereby converted to succinate and carbon dioxide (Fig. 7). Desulfonation of taurine by TauD requires equimolar amounts of α-ketoglutarate as cosubstrate, and though α-ketoadipate also leads to a low level of desulfonation, other α-ketoacids are not accepted as cosubstrates. TauD is quite specific for taurine as substrate, and though it will also desulfonate short-chain alkanesulfonates (C_4–C_6), the K_m values are an order of magnitude higher than for taurine [12].

Known α-ketoacid-dependent dioxygenases catalyze reactions of a variety of substrates, including 2,4-dichlorophenoxyacetic acid (2,4-D), the amino acids lysine, aspartate, proline and γ-butyrobetaine, gibberellins, β-lactams, flavonoids and alkaloids [219,220]. They are found in plants, animals and bacteria, and carry out not only classical hydroxylation reactions, but also desaturations and ring expansions. Sequence comparison of the characterized enzymes show that they make up a diverse group that probably arose by convergent evolution, rather than from a common ancestor. This is reflected in the varied structure of members of the enzyme family. The subunit sizes vary from 26 to 85 kDa, and the native enzymes are monomers or homodimers (though one $\alpha_2\beta_2$-heterotetramer is known) [220]. TauD is a homodimer with subunit molecular mass of 32 kDa. At a sequence level it is related to the dichlorophenoxyacetate dioxygenase encoded on plasmid pJP4 of *Ralstonia eutropha* (30% amino acid identity) [221], though the two enzymes do not react with each other's substrates [12]. TauD does not show significant sequence similarity to other characterized α-ketoacid-dependent dioxygenases, including bacterial enzymes of this family.

However, comparison of *tauD* with sequences arising from various genome sequencing projects indicates that close relatives of the *tauD* gene are not uncommon. Thus, open reading frames of unknown function which are related to *E. coli* TauD have been identified in *Yersinia pestis* (70% sequence identity to TauD [144]), *Saccharomyces cerevisiae* (32% identity; accession number Z47973), *M. tuberculosis* (36% identity; accession number Z77165), *B. pertussis* (three ORFs, with 32%, 39% and 48% sequence identity [144]), and *P. aeruginosa* (three ORFs, with 37%, 40% and 60% sequence identity [122]). In *P. aeruginosa* one of the three *tauD* homologues can definitely be assigned to *tauD* since it occurs in the same operon structure as *tauD* does in *E. coli*; an α-ketoglutarate-dependent taurine dioxygenase activity has also been observed in this strain (unpublished results). The second *tauD* homologue is also regulated as part of the sulfur cycle, since it has been identified as an SSI protein (protein PA4 [20]). The third homologue is likely to be similarly regulated, since it is located adjacent to the gene encoding the PA4 protein. In the absence of biochemical studies of these proteins, it is not yet clear if they are all active as α-ketoglutarate-dependent dioxygenases, and if so, with what substrates. However, the presence of three such genes, all induced under sulfate limitation conditions, suggests that the corresponding proteins may well play a role in organosulfur metabolism.

5.2. Desulfurization of methanesulfonate and other alkanesulfonates; $FMNH_2$-dependent monooxygenases

E. coli strains that have been mutated in the *tauD* gene are no longer able to grow with taurine as sole sulfur source, but their growth with a variety of other sulfonates is unimpaired. At least one other desulfonation system must therefore exist in the cell. This system has been identified as the *ssu* operon, which has been characterized in *E. coli* [222], *P. putida* [132], and *B. subtilis* [223] (Fig. 8).

Synthesis of the *ssu* gene products is repressed in the presence of inorganic sulfate, and indeed they were originally identified in *E. coli* [22] and *P. aeruginosa* [20,24] by differential 2D-PAGE in a screen for sulfate starvation-induced proteins.

Desulfonation of alkanesulfonates is catalyzed by the products of the *ssuD* and *ssuE* genes, which encode an $FMNH_2$-dependent monooxygenase and an NAD(P)H-dependent FMN reductase, respectively (Fig. 8). In all species so far studied, the *ssu* operon also contains genes encoding a putative ABC-type transport system that presumably catalyzes uptake of alkanesulfonates into the cell. In the pseudomonads, but not in *E. coli* or *B. subtilis*, the distal gene in the operon (*ssuF*) encodes a small protein related to putative molybdopterin binding proteins of *C. pasteurianum*, but the significance of this is not yet clear (molybdenum does not appear to be required for growth with sulfonates [132]). *P. aeruginosa* also contains a second operon (*msuEDC*) comprising closely related copies of the sulfonatase and reductase genes, which encodes a methanesulfonate sulfonatase system [14]. Although PCR and Southern analysis showed that the *ssuD* gene is widespread among pseudomonads [14], in the bacterial genomes that have been completely or partially sequenced to date further *ssuD* homologues have been found only in *M. tuberculosis* and in *Y. pestis*.

The *ssuD* gene product is most closely related to the monooxygenase components of two-component enzyme systems that catalyze the oxygenation of EDTA [224], nitrilotriacetate (NTA) [225], pristinamycin II_B [226] and dibenzothiophene dioxide [15,227] (the latter three are encoded by the *ntaA, snaA* and *dszA* genes, respectively). These monooxygenases are characterized by their requirement for reduced FMN supplied by a second, NAD(P)H-dependent FMN reductase component, and the enzymes themselves do not contain a bound flavin molecule. This use of reduced flavin as a cosubstrate, rather than as a bound prosthetic group, is a significant difference from other characterized mono- and dioxygenases, including the mammalian flavin monooxygenases. Studies with the NTA and EDTA oxygenase enzyme systems [224,225], and with MsuED [14], suggest that the flavin reductase

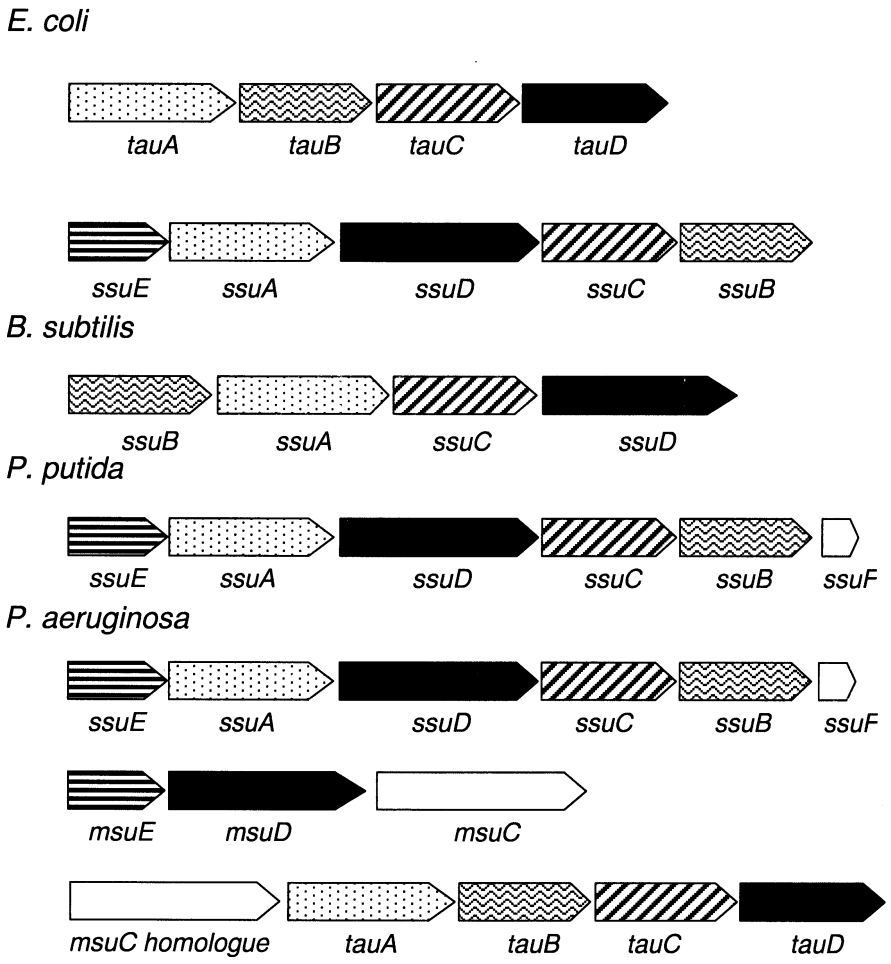


Fig. 8. Genetic organization of bacterial sulfonatase-encoding operons. The enzymes encoded in each gene cluster are putative oxygenases (■), NADH-dependent FMN reductases (▤) and the components of ABC-type transporters: periplasmic solute binding proteins (⁝⁝), ATP binding proteins (▨) and permease proteins (t). No definite function is known for the products of the *ssuF* and *msuC* genes [14,132]. The genes are from: *E. coli* [218,222], *B. subtilis* [223], *P. putida* [132,185], and *P. aeruginosa* [14,122].

component may be associated with the monooxygenase as part of a loose complex (the two components co-purified over anion exchange, but were dissociated during further purification steps). No evidence for complex formation was found for the dibenzothiophene system [15]. In addition, the flavin reductase enzymes of the NTA and EDTA oxygenases were interchangeable, and could also be replaced by the flavin reductase from *Vibrio fischeri* [224]. It therefore appears that the flavin reductase enzymes are not essential parts of a complex, but that their role is limited to providing reduced FMN for the oxygenase component.

This conclusion is also supported by the genetic organization of the $FMNH_2$-oxygenases and their cognate flavin reductases. Whereas the reductase and oxygenase components of the *P. aeruginosa* and *P. putida* sulfonate-desulfurizing systems (SsuED and MsuED) are each co-expressed as part of an operon (*msuE* and *msuD* even overlap by two base pairs), this organization is the exception. In *Rhodococcus erythropolis* the *dszD* flavin reductase gene is unlinked to the *dszABC* genes, in *S. pristinaespiralis snaA* and *snaC* are separated by at least 24 kb, and in *Chelatobacter* the *ntaA* and *ntaB* genes, though adjacent, are divergently oriented [225]. The operon containing the *B. subtilis ssuD* gene lacks a flavin reductase gene (Fig. 8) [223]. Analysis of the genome sequence did not reveal any gene products related to known flavin reductases such as the Fre protein of *E. coli* [228], MsuE/SsuE genes of *E. coli* or pseudomonads [14,24,132], or the DszD protein of *R. erythropolis* [15], accession number AF048979). The *B. subtilis* FMN reductase must therefore belong to another, hitherto uncharacterized family of flavin reductase enzymes. The monooxygenases described fall into two families, with the SsuD/MsuD enzymes 60–92% identical to each other, and the NtaA/SnaA/DszA group 39–46% identical, with about 20% identity between the two groups. Sequence comparison of the cognate reductases shows that these are much less related, and can be divided into at least three groups, with less than 15% identity between groups.

The products of the *P. aeruginosa msuD* and *msuE* genes have recently been overexpressed in *E. coli* and partially characterized. They catalyze the desulfonation of methanesulfonate and, at a lower rate, several related alkanesulfonates [14]. The monooxygenase gene *msuD* is closely related to the *ssuD* genes, and appears to be redundant in *P. aeruginosa*, since an *msuD* mutant was able to grow with all sulfur sources tested, including a range of sulfonates. This functional redundancy has been ascribed to the *ssuD* gene – although no detailed studies of *ssuD* have yet been made in *P. aeruginosa*, the *ssuD* gene of *P. putida* has been shown to be required for methanesulfonate utilization in that species [132]. In contrast, *E. coli* K12, despite possessing a closely related *ssuD* gene (77% identity at protein level) is unable to grow with methanesulfonate as sulfur source (though *E. coli* B can do so), and the overexpressed SsuD enzyme shows very low activity with methanesulfonate as substrate (E. Eichhorn, personal communication). This suggests that the *E. coli* SsuD protein contains an altered active site that cannot bind methanesulfonate, or that an additional essential factor for this reaction is present in the pseudomonads which is lacking in the enteric species.

The function of the third gene in the *msu* operon, *msuC*, is less well defined. The presence of excess MsuC leads to an increase in the rate of MsuD-catalyzed desulfonation of methanesulfonate, but MsuC itself does not catalyze the desulfonation reaction [14]. The *msuC* gene product is quite similar (42% identity) to another $FMNH_2$-dependent monooxygenase, the enzyme that carries out the initial step in the dibenzothiophene (DBT) desulfurization pathway in *R. erythropolis* IGTS8 (see below). This enzyme catalyzes the stepwise oxidation of DBT to DBT-dioxide, and because of the similarity of the MsuC and DszC enzymes it is tempting to speculate that the natural substrates of MsuC in *P. aeruginosa* may therefore be organosulfides. Oxidation of these to the corresponding sulfonates would then provide suitable substrates for the cognate sulfonatase. However, *P. aeruginosa* is unable to grow with either aromatic or aliphatic sulfides as sulfur sources, so MsuC presumably plays another role. Both MsuC and DszC are related to the family of eukaryotic short-chain-specific acyl-coenzyme A dehydrogenases (24–25% identity over nearly the full length of the protein). Although this has been commented on by several authors [15,229], its significance is still unclear. The glutamate res-

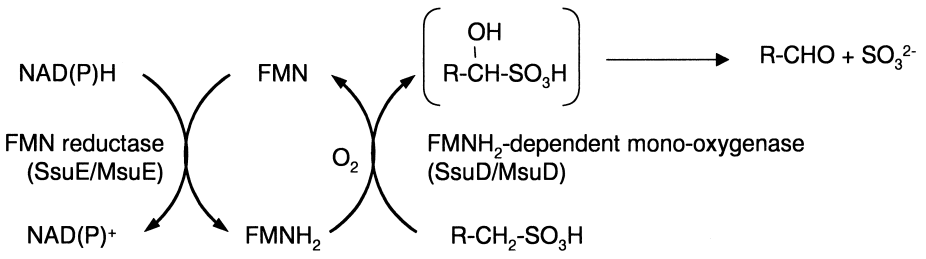


Fig. 9. Proposed mechanism for the $FMNH_2$-dependent oxygenolytic desulfonation of alkanesulfonates. FMN is reduced by the FMN reductase, using either NADH or NADPH as electron donor. The reduced FMN acts as a cosubstrate in desulfonation by the SsuD or MsuD proteins, yielding the α-hydroxysulfonate which decomposes to the product aldehyde with release of sulfite.

idue which is required for acyl-CoA dehydrogenase activity [230,231] is not conserved in MsuC or DszC. Possibly MsuC plays a role in flavin binding – reduced flavin bound to acyl-CoA dehydrogenases has been shown to be resistant to oxidation by molecular oxygen [231,232], and this may have relevance for stabilization of the flavin reducing equivalents required in the monooxygenase reactions.

The importance of sulfonates within the natural bacterial life cycle has only been recognized recently, and is exemplified by the fact that in the organisms in which sulfur-controlled sulfonate metabolism has been studied, two or even three discrete desulfonation systems have been found, with overlapping substrate ranges. Many soil bacteria can utilize sulfonates as sulfur source [14,209,213], as can a number of enteric bacteria [210]. Although it seems odd that enteric bacteria should contain several sulfate-repressed desulfonative oxygenase systems, since the gut contains high levels of sulfate and is largely anaerobic, these genes may be crucial for survival outside the gut environment. Further studies are still required to determine whether the genes responsible for alkanesulfonate uptake and desulfonation in enteric bacteria are regulated entirely by sulfur supply in the gut, or whether they also respond to other environmental stimuli.

6. Desulfonation of aromatic sulfonates – a further adaptation

Like aliphatic sulfonates, aromatic sulfonates can in principle provide bacteria with either sulfur or carbon for growth. The enzyme systems required in both cases are oxygenases. Desulfurization of aromatic sulfonates uses the same oxygenase as for their aliphatic counterparts, though an additional electron transport chain is required. Full mineralization proceeds by pathways that are similar to well known routes for the degradation of other aromatic compounds.

Desulfonation of aromatic sulfonates and ring degradation to provide carbon for bacterial growth can proceed by several pathways, which have recently been reviewed [7,233]. Desulfonation usually occurs prior to ring cleavage [234–236], but can also take place after ring cleavage [237–239] or even simultaneously with ring cleavage, as in the degradation of 2-aminobenzenesulfonate [190]. The desulfonatase enzymes that have been characterized are multicomponent dioxygenase systems that are induced in the presence of their substrates. Many of these are plasmid-encoded [240,241], thus facilitating their horizontal transfer between species.

Bacteria are also capable of growing with aromatic sulfonates as sulfur source, even when they do not further metabolize the carbon skeleton of the compounds. Both the biochemistry and the regulation of sulfur-regulated systems are quite different from those which mediate arylsulfonate utilization as a carbon and energy source. Three independent studies have reported that under sulfate-limiting conditions arylsulfonates are desulfonated to the corresponding phenols, in *P. putida* S-313 [242], *Klebsiella oxytoca* KS3D [243] and the mixed culture L6 [244]. $^{18}O_2$-incorporation experiments with the *P. putida* and *K. oxytoca* strains showed that the phenol oxygen atom was derived from molecular oxygen, and that the reaction is therefore catalyzed by a monooxygenase. The substrate range of this enzyme is very wide indeed – *P. putida* S313 can desulfonate several hundred different aromatic sulfonates [7], whereas the L6 culture was able to desulfonate the complex mixture of compounds that is present in commercial LAS [244]. Because of this substrate tolerance, the *K. oxytoca* system has been considered for the industrial synthesis of phenols that are inaccessible by traditional methods [243].

However, does this catalytic proficiency arise from the action of one enzyme or of a family of related enzymes? Transposon mutagenesis of *P. putida* S-313 yielded several groups of mutants which were deficient in growth with benzene sulfonate, representatives of which are shown in Table 4. One of these mutants (strain SN34) was unable to grow with a variety of organosulfur sources, including alkane and arenesulfonates, aromatic and aliphatic sulfate esters, methionine, and methionine biosynthetic intermediates such as homocysteine or cystathionine, though it grew normally with inorganic sulfate. This strain carried a transposon insertion in the *ssuE* gene, and complementation analysis with constructs carrying in-frame deletions in

Table 4
Growth of *P. putida* S-313 and derivatives with various organosulfur sources

Strain	Genotype	Growth[a] with:			
		aromatic sulfonates	aliphatic sulfonates	aromatic sulfate esters	aliphatic sulfate esters
S-313	wild-type	+	+	+	+
SN34	*ssuE*::miniTn*5*	−	−	−	−
SN34/pME4433	Δ*ssuD*	−	−	−	−
SN34/pME4431	Δ*ssuE*	−	+	+	+
SN57	*atsB*::miniTn*5*	−	+	−	−
PW15	*atsR*::miniTn*5*	+	+	−	−
SN36	*asfA*::miniTn*5*	−	+	+	+

[a]Organosulfur compounds were supplied as sole sources of sulfur for growth, at a concentration of 100–500 μM.

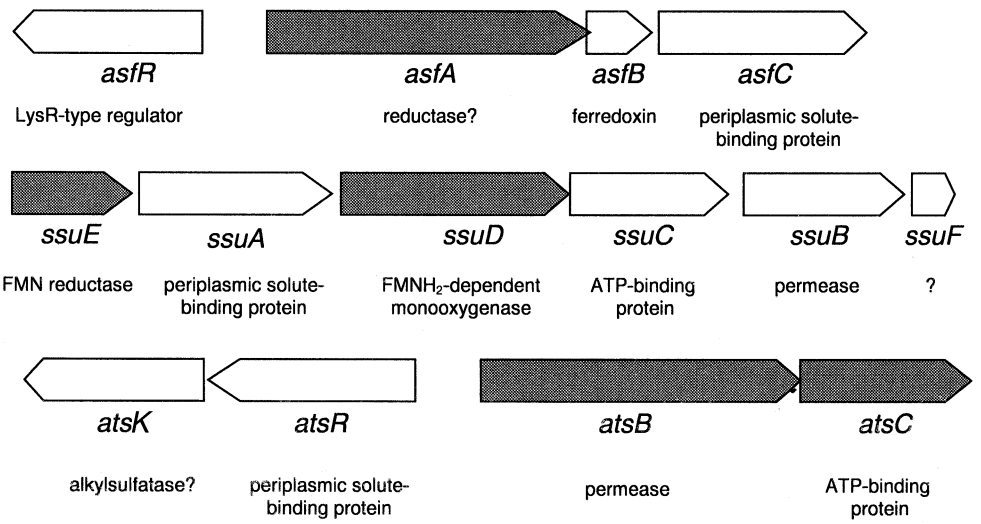


Fig. 10. Genes required for growth with aromatic sulfonates in *P. putida* S-313. Three separate gene clusters (*asf, ssu* and *ats*) were identified by transposon mutagenesis and selection for desulfonation-negative mutants [185]. Putative functions of each gene product are shown. The shaded genes are those that are required for growth with aromatic sulfonates [132,185].

the individual genes of the *ssu* operon showed that aromatic desulfonation required intact *ssuD* and *ssuE* genes. These encode the flavin reductase and $FMNH_2$-dependent monooxygenase that were previously implicated in alkanesulfonate metabolism in this species, suggesting that the same monooxygenase system may be responsible for desulfonating not only alkanesulfonates, but also the full range of aromatic sulfonate substrates.

Many organisms can grow with alkanesulfonates as sulfur source, and the *ssuD* gene is widespread. However, very few of these organisms can use aromatic sulfonates as sulfur source. Therefore, either the *P. putida* SsuD protein is specifically adapted to allow desulfonation of aromatic substrates, or a factor is required for the aromatic desulfonation which is not needed for desulfurization of alkanesulfonates. Characterization of a second group of non-desulfonating mutants of *P. putida* S-313 led to the discovery of the *asf* operon (Fig. 10), which appears to be the key locus in controlling arylsulfonate utilization in this strain. It has been described as a 'toolbox' for arylsulfonate utilization [185], since it encodes a putative arylsulfonate binding protein (AsfC), an electron transport system (putative reductase (AsfA) and ferredoxin (AsfB)) and a divergently oriented LysR-type regulator (AsfR), but not the desulfonating oxygenase. When *P. aeruginosa* PAO1 was transformed with the *asfABC* genes behind a *lac* promoter, it gained the ability to grow with a variety of arylsulfonates, consistent with the hypothesis that the *asf* genes are the additional factor required to enable the SsuD protein to desulfurize aromatic sulfonates. A working model consistent with the current data is shown in Fig. 11.

The gene products of the *asf* operon have not yet been characterized biochemically, but their reconstitution promises to generate a novel, multicomponent monooxygenase system. Most monooxygenases either do not have a free ferredoxin component at all, or they contain a [2Fe-2S] plant-type ferredoxin. The three-component methanesulfonate monooxygenase of *M. methylovora* M2, for example, contains a Rieske-type [2Fe-2S] ferredoxin [194], which is related to the ferredoxin components of benzene and toluene monooxygenase systems. By contrast, the 12-kDa ferredoxin encoded by *asfB* contains two [4Fe-4S] clusters in its N-terminal domain. Ferredoxins of the [4Fe-4S] family are known in anaerobic organisms, where they are involved in coupling substrate oxidation (e.g. formate, pyruvate) to reduction of an electron acceptor (e.g. NAD^+, $NADP^+$, FMN, FAD), and in photosynthetic organisms [245]. The gene product of *asfA* is similar to the flavoprotein subunit of fumarate reductases from several species (24–27% identity). The role of AsfA and AsfB is not yet clear – whereas they may play a part in energizing the uptake of aromatic sulfonates into the cell, it seems more likely that they accept reducing equivalents from $FMNH_2$ (especially since *asfA* is related to the flavoprotein fumarate reductase), and transfer them to the oxygenase. Reduced FMN is highly unstable under aerobic conditions, as it is rapidly reoxidized by molecular oxygen, with concurrent release of damaging superoxide radicals [246]. The AsfAB system may therefore provide a mechanism for stabilizing reduced flavin equivalents such that they can subsequently be used to supply electrons for the aromatic desulfonation reaction (see Fig. 11).

A third locus is also required for aromatic desulfonation in *P. putida* S-313. Mutants in the *atsB* gene were not only defective in utilization of aromatic and aliphatic sulfate esters, as described above for *P. aeruginosa*, but were also unable to grow with aromatic sulfonates. This suggests that the uptake system encoded by *atsRBC* transports a broad range of sulfated and sulfonated substrates

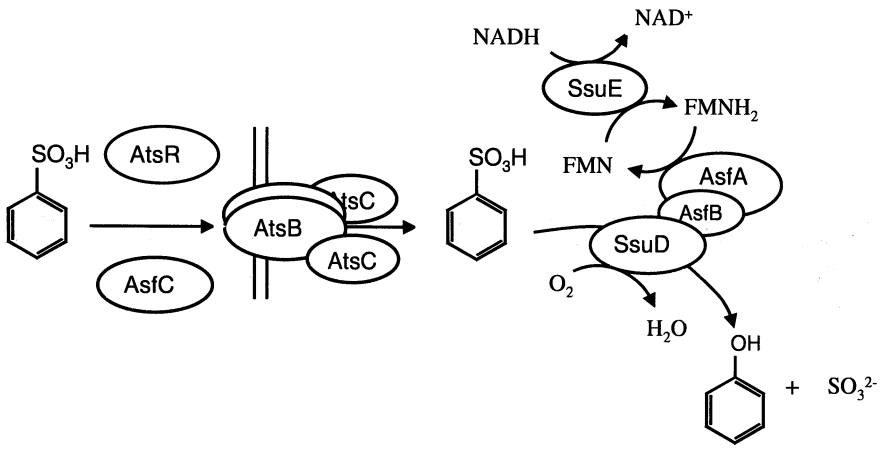


Fig. 11. Working model of aromatic desulfonation in *P. putida* S-313. Uptake of aromatic sulfonates into the cell is catalyzed by the AtsBC system, together with the solute binding proteins AsfC or AtsR. The desulfonation reaction is carried out by the $FMNH_2$-dependent SsuD, with reduced flavin supplied by the flavin reductase encoded by *ssuE*. The role of AsfA and AsfB is not yet clear, but they may play a role in transferring reducing equivalents to the oxygenase [185]. The subunit stoichiometries in the desulfonation complex have not yet been determined.

into the cell, though biochemical studies to confirm this have not yet appeared. A *P. putida* mutant in *atsR* was deficient in desulfurization of sulfate esters, but could grow with aromatic sulfonates, presumably because of the presence of the *asfC* gene in this organism. This was confirmed by the fact that aromatic desulfonation in a wild-type *P. aeruginosa* strain required the presence of only the *asfAB* genes, but in an *atsR* mutant the *asfC* gene product was also needed [185].

Although aromatic sulfonates have largely been regarded as xenobiotic compounds in the past, bacterial isolates that can desulfonate them can be readily isolated from natural environments. Enrichment cultures with a variety of such compounds (ranging from the simplest representative, benzenesulfonate, to complex sulfonated dyes) have yielded desulfonating isolates not only with inocula from wastewater treatment works, but also from environments with lower xenobiotic exposure, such as forest and garden soils, and rumen contents [252]. The strains obtained in this study were predominantly *Pseudomonas* species, though this may in part simply reflect the isolation strategy applied. Analysis of these desulfonating strains revealed the presence of a highly conserved *ssuD* gene in most of them ($>90\%$ amino acid identity). An *asfA* gene was found in all the *P. putida* isolates, but not in other strains. If AsfAB indeed constitutes an electron transport system delivering electrons to the monooxygenase in *P. putida*, then this function must be fulfilled by other proteins in other species. In this context, it is interesting to note that *P. aeruginosa* also carries an *asfABC* homologue in its genome, even though it cannot grow with aromatic sulfonates. The *P. aeruginosa* AsfA homologue is 29% identical to the *P. putida asfA* gene product. The two ferredoxins are even more closely related (35% identity), and though the *P. aeruginosa* AsfB is somewhat smaller than the *P. putida* counterpart, it also contains two putative [4Fe-4S] clusters.

Two of the loci involved in aromatic desulfonation, *ats* and *ssu*, have been shown in *P. aeruginosa* to be upregulated during growth with a variety of sulfur sources, and repressed in the presence of sulfate, cysteine or thiocyanate, and they therefore represent typical sulfate starvation-induced systems [20,24,136]. The *asf* operon, by contrast, is also controlled by its own substrate. Expression studies with *asfA* and *asfR* showed that AsfR was a negative regulator of the *asfABC* operon, and that in addition, *asfR* was subject to positive autoregulation [185]. In the presence of toluenesulfonate, *asfR* expression was repressed, and the resulting fall in cellular AsfR levels apparently derepressed the *asfABC* genes, since expression of *asfA* increased dramatically during growth with toluenesulfonate. Interestingly, the toluenesulfonate-mediated repression of *P. putida asfR* expression appeared to be partly independent of the AsfR protein, since it was also observed with a *P. putida asfR::xylE* fusion in *P. aeruginosa*, in the absence of a functional *asfR* gene. This suggests the presence of an additional, unidentified factor in this response. During growth with toluenesulfonate and sulfate, *asfA* expression was repressed, and this effect was found to be at least in part mediated by the global control protein CysB [185].

Under sulfate limitation conditions, *P. aeruginosa* synthesizes increased levels of two antioxidant proteins, a putative thiol-specific antioxidant (TSA; also called a thiol peroxidase), encoded by the *lsfA* gene (accession number AF075709), and the alkylhydroperoxide reductase AhpC [20,24]. The latter protein is also upregulated during sulfate starvation in *E. coli* [22]. The *lsfA* gene is located immediately upstream of the *ssu* operon in *P. putida*, and 3.0 kb upstream of *ssuE* in *P. aeruginosa* (in *P. putida lsfA* and *ssuE* are transcribed separately, but in *P. aeruginosa* this has not yet been examined). LsfA and AhpC show 30–65% sequence identity to a number of antioxidant proteins grouped together as the TSA/AhpC family

[247], and the *P. aeruginosa* LsfA protein, which has recently been overexpressed and purified, indeed displays the anticipated thiol-specific antioxidant activity [248]. The relevance of these antioxidant proteins in the sulfate starvation response may be linked to sulfonate utilization. In the absence of sulfonated compounds, induction of *ssuE* (and *msuE* in *P. aeruginosa* [14]) by sulfate starvation will lead to an excess of reduced flavin nucleotides in the cell, and consequently to an increase in radical-caused damage. Increased synthesis of LsfA and AhpC under sulfate-limited conditions may represent the cell's response to this threat. However, since the cell's main defense to oxidative stress consists of superoxide dismutase and catalase activities, which are not regulated by the sulfur supply to the cell, it is not clear why an increase in LsfA and AhpC levels should be necessary.

LAS is the best investigated of the arylsulfonates from a degradation standpoint. Commercial LAS is a mixture of many different isomers and homologues that are a degradative challenge for bacterial populations, especially under anaerobic conditions and in sediments, where significant levels of LAS can accumulate (e.g. [65]). The proposed mechanism by which LAS is mineralized was first proposed by Willetts and Cain [249], and evidence for it has been reviewed by Swisher [60] and Schöberl [250]. Degradation is initiated by the oxidation of the methyl group on the alkane chain that is more distant from the aromatic ring (the so-called distance principle [60]), followed by a series of β-oxidation steps that give rise to a sulfophenylalkanoate compound such as sulfophenylbutyrate. Further degradation of this aromatic sulfonated intermediate proceeds via oxygenative desulfonation and ring cleavage, and probably occurs in a separate organism from the initial β-oxidation steps [251]. Commercial LAS surfactants are generally regarded as being readily biodegradable [67,252,253], although impurities generated as byproducts in the industrial synthesis (e.g. dialkyltetralinsulfonates, DATS) may be more of a problem ([253,254], but cf. [255]).

Direct desulfonation of complex LAS surfactant mixtures has also been observed [244] when LAS was supplied only as a sulfur source for bacterial growth. It yielded the corresponding long-chain-substituted phenols. Interestingly, the selectivity of this sulfate-limited desulfonation process is exactly reversed to that observed during carbon-limited degradation. Under carbon-limited conditions, longer-chain isomers are degraded more rapidly, and there is a preference for positional isomers in which the sulfophenyl ring is near the end of the chain [60]. Under sulfur-limited conditions the reverse is true, as the short-chain isomers are preferentially desulfonated, and centrally substituted isomers are degraded faster than 2-substituted LAS [244]. In the presence of inorganic sulfate no direct desulfonation occurs [244], and so although this reversed selectivity has possible potential in wastewater treatment, a different genetic control must first be introduced, since wastewaters commonly contain high levels of inorganic sulfate. It is to be hoped that genetic studies such as the above will lead to an understanding of the mechanism by which these genes are repressed in the presence of sulfate, and development of methods to circumvent this for practical applications.

7. Biodesulfurization of condensed thiophenes

The desulfurization of fossil fuels continues to be the subject of intensive research. Sulfur constitutes up to 5% (w/w) of crude oils, and most of it is present in organically bound form [256]. On combustion, organosulfur compounds are converted to corrosive sulfur oxides, which return to earth in acid rain. As legislative limits to sulfur emissions become tighter, the need to remove the organosulfur from fuel has become more pressing, and efforts to develop biodesulfurization methods to replace the current hydrodesulfurization process have increased (see the excellent recent reviews by McFarland and coworkers [52,257]).

More than 60% of the sulfur in higher boiling fractions of crude oil is present as DBT and substituted dibenzothiophenes [256], and DBT has therefore been the model compound of choice for investigating the molecular basis of biodesulfurization. DBT can be used as a carbon source by a number of Gram-negative species, including several pseudomonads (e.g. [258,259]) and a *Rhizobium meliloti* isolate [260], but in most cases DBT conversion occurs co-metabolically during growth with another carbon source [256]. DBT biodegradation under these conditions takes place via dioxygenase attack on the benzene ring, followed by *meta* cleavage of the resulting dihydroxy-DBT, and oxidative decarboxylation to 3-hydroxy-2-formylbenzothiophene via the 'Kodama' pathway [261]. In pseudomonads this pathway is plasmid-encoded [262]. The dioxygenase carrying out the initial reaction in *Pseudomonas* sp. strain C18 (a DBT-metabolizing soil isolate) was identical with *P. putida* naphthalene dioxygenase [263], and was required for metabolism not only of DBT, but also of naphthalene and phenanthrene. Purified naphthalene dioxygenase indeed catalyzes dioxygenation of DBT, but can also oxidize the DBT sulfur atom to the sulfoxide [264].

However, although DBT metabolism via the Kodama pathway leads to a reduction in the toxicity of the starting compounds, it does not remove the sulfur, and it involves some loss of carbon, thereby reducing the fuel value of the product. Bacteria that can remove the sulfur atom from DBT without attacking the carbon backbone have been isolated by selecting for strains that could utilize DBT as a sulfur source for growth. The isolates obtained have been almost exclusively rhodococci [265–272] and other Gram-positive species [273–275], including two thermophilic *Paenibacillus* strains [276]. Under sulfur-limiting conditions, desulfurization of DBT proceeds via the path-

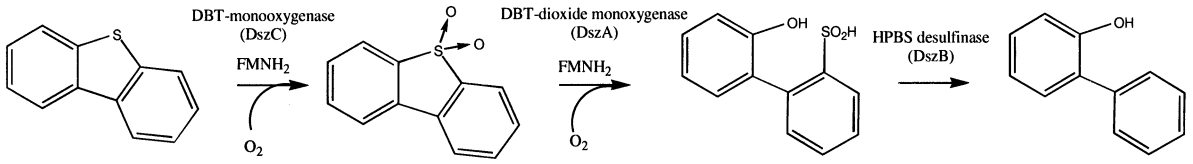


Fig. 12. Desulfurization of dibenzothiophene by the *R. erythropolis* IGTS8 pathway. The thiophene sulfur is oxidized in two steps by DBT-monooxygenase to DBT-dioxide, which is then cleaved by DBT-dioxide monooxygenase to 2-(2-hydroxyphenyl)benzenesulfonate (HPBS). The sulfinate group is finally released by HPBS sulfinase. Reduced FMN for the two oxygenolytic steps is provided by the FMN reductase DszD. Adapted from [15].

way shown in Fig. 12. The enzymes concerned have been characterized from *R. erythropolis* IGTS8 [15], but the pathway appears to be very similar in all organisms studied, including two Gram-negative organisms [277,278]. DBT is initially oxidized in two steps to DBT-*S*,*S*-dioxide by DBT monooxygenase (the product of the *dszC* gene). The first C-S bond is then cleaved oxygenolytically, by DBT-*S*,*S*-dioxide monooxygenase (DszA), and the desulfurized product is released after reductive removal of the resultant sulfinate moiety by a novel desulfinase enzyme (DszB). The genes encoding these enzymes are encoded in the *dszABC* operon. Expression of this operon is repressed during growth with sulfate, cysteine or methionine [271,279] but derepressed in the presence of DMSO or DBT. Further analysis is required to determine whether expression of the *dsz* genes reflects a general response to sulfate limitation, or whether they are only upregulated in the presence of a suitable inducer (as seen for e.g. the arylsulfonate degradation system *asfABC* [185]). Band-shift analysis of the *dsz* promoter region revealed that a putative repressor protein may play a role in the downregulation observed with sulfate [279], but this has not yet been explored further.

The two oxygenases in this pathway both require reduced FMN for activity, and the pathway is therefore clearly related to that for the cleavage of sulfonates reported above. Indeed, the second step in DBT desulfurization, cleavage of the thiophene C-S bond to yield hydroxybiphenylsulfinate (Fig. 12), resembles an intramolecular version of the aromatic sulfonate desulfonation found in *P. putida*. Despite the apparent similarity in the reaction mechanisms of this initial C-S cleavage reaction, the DszA protein is only 26% identical to the SsuD protein of *P. putida*, and the two systems are also catalytically distinct from each other; *P. putida* cannot desulfurize DBT [185], and a *R. erythropolis* strain which was able to grow with aromatic sulfonates and contained the *ssuD* gene did not grow with DBT [280]. Like other oxygenases of this family [14,224,226,281], the DszA and DszC oxygenases do not contain a tightly bound flavin cofactor [15]. Instead, they use $FMNH_2$ as a cosubstrate. Reduction of FMN to provide $FMNH_2$ is catalyzed by an NADH-dependent FMN reductase (DszD) [15], but this can be replaced in vitro by other FMN reductases [282]. The 25-kDa DszD protein does not contain bound flavin, unlike the related FMN reductases involved in EDTA, NTA and pristinamycin metabolism [224,226,281] (33–37% identity). In rhodococci, the *dsz* operon is carried on a plasmid 100–150 kb in size (pSOX) [229,283,284], and is associated with putative insertion sequence elements, IS*1166* and IS*1295* [285]. The DszD protein is not encoded with the rest of the *dsz* operon, and it will be interesting to discover whether it is also encoded on the pSOX plasmid, or whether the *dszD* gene is chromosomally located.

Most of the DBT-degrading organisms studied are Gram-positive, and it has been speculated that the desulfurization pathway observed in strain IGTS8 (Fig. 12) might even be *Rhodococcus*-specific, due to a requirement for an additional, as yet unidentified factor [283,284]. However, DBT desulfurization under sulfate-limiting growth conditions has also recently been reported for two Gram-negative organisms, *Agrobacterium* sp. strain MC501 [277] and *Sphingomonas* sp. strain AD109 [278]. Both these organisms used the same pathway for DBT degradation as strain IGTS8, yielding hydroxybiphenyl as the desulfurized product. The *dsz* genes of *Sphingomonas* sp. AD109 were found to have the same organization as in *Rhodococcus* sp. IGTS8, and the deduced proteins were also found to be closely related to the rhodococcal enzymes (67–76% identity). In addition, desulfurization of DBT has recently been demonstrated in specifically designed recombinant *Pseudomonas* strains [286]. The *dszABC* genes of *R. erythropolis* IGTS8 were cloned behind the *tac* promoter and introduced into *P. putida* and *P. aeruginosa* either on the broad-host-range plasmid pVLT31 or in single copy using a mini-Tn*5* derivative. The resultant strains grew more quickly than *R. erythropolis* IGTS8 with DBT as sole sulfur source, and converted DBT quantitatively to hydroxybiphenyl [286]. Since *Pseudomonas* strains are quite solvent-tolerant [287], this approach promises to provide useful strains for desulfurization applications. Use of the constitutive *tac* promoter also alleviates the most pressing problem in industrial biodesulfurization applications, that the system is repressed in the presence of sulfate.

8. Regulation of bacterial organosulfur metabolism

One of the main discoveries to arise from recent studies on organosulfur metabolism in Gram-negative bacteria is that the CysB protein, whose main characterized role was

in assimilation of inorganic sulfate, is also required for expression of systems involved in transport and metabolism of sulfate esters and sulfonates both in enteric bacteria and in pseudomonads, and hence controls sulfur metabolism on a larger scale. CysB of *E. coli* is a homotetramer consisting of 36-kDa subunits encoded by the *cysB* gene. It is a member of the LysR family of transcriptional activators, a large class of proteins which carry a helix-turn-helix DNA binding motif in the N-terminal domain [288] (residues 19–38 of CysB). CysB acts as a class I transcriptional activator, binding to activation sites located just upstream of the −35 region of the *cysJ, cysK* and *cysP* promoters and presumably interacting with the carboxy-terminal portion of the RNA polymerase α subunit [289]. In the presence of *N*-acetylserine, CysB activates transcription from the above promoters; the initial binding of the CysB protein to the DNA does not require the presence of *N*-acetylserine, but the presence of the co-inducer stimulates the binding in specific ways at the different *cys* promoters [5]. For the closely related *K. pneumoniae* CysB protein a dissociation constant of 4 mM has been reported for *N*-acetylserine [290], though this seems remarkably high for a regulatory molecule. CysB also binds directly to its own promoter, where it acts as a repressor, and to several other sites upstream of the activated promoters. Consensus binding sites for CysB have been defined in *E. coli* and *S. typhimurium* in a number of detailed studies by the group of Kredich (summarized in [5]), but the consensus sequence is relatively poorly conserved, and does not allow confident prediction of further sites of activation by CysB. This is unfortunate, because recent work has shown that CysB is a truly global regulator − in addition to regulating cysteine biosynthesis, and organosulfur metabolism, it is also involved in acid resistance in *E. coli* [291,292], in alginate biosynthesis in *P. aeruginosa* [293], and in pectinolysis and cellulolysis in *Erwinia carotovora* [294].

The most obvious feature that links the expression of genes involved in cysteine biosynthesis and those of organosulfur metabolism is that they require sulfate-limited conditions for full expression. The basis of this regulation has been investigated in detail for the sulfate assimilation pathway in enteric bacteria, and is also CysB-mediated. Two main levels of control exist. Firstly, the endproduct of the pathway, cysteine, exerts feedback inhibition on serine transacetylase, which converts serine to *O*-acetylserine (Fig. 1). This controls the levels of acetylserine in the cell, and hence the level of CysB-activated transcription (Fig. 13). A second level of control is mediated by sulfide and thiosulfate (Fig. 13), which act as anti-inducers of the *cys* regulon, probably competing with acetylserine for binding to the CysB-DNA complex [295]. CysB was found to be very similar in structure to the sulfate binding protein, with a binding pocket where either acetylserine or thiosulfate are thought to bind as co-inducer and anti-inducer, respectively [295]. Sulfate does not itself act as an anti-inducer, so it was interesting that the crystal structure revealed a sulfate anion bound in the substrate pocket. The relative contributions of cysteine-mediated inhibition of serine transacetylase and sulfide/thiosulfate anti-induction in controlling the *cys* regulon have been assessed in *S. typhimurium*. Sulfide repressed expression of the *cysDNC* operon (encoding the proteins that catalyze sulfate activation to PAPS) almost as well in a *cysK cysM* background as in the wild-type strain. Since the *cysK cysM* strain is unable to convert sulfide to cysteine, this suggests that anti-induction by sulfide is the main regulatory factor in this species [296].

The genes involved in sulfonate metabolism in *E. coli*, *tauABCD* and *ssuEADCB*, were initially identified either

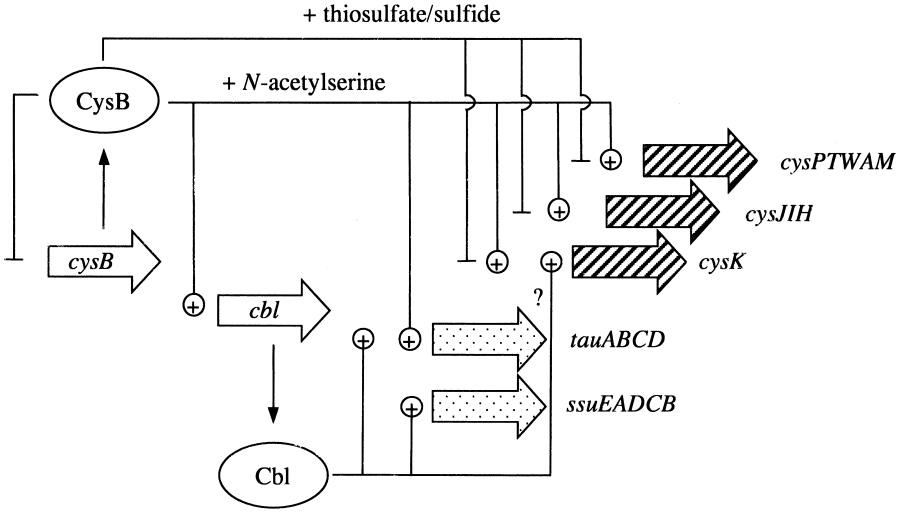


Fig. 13. Regulation of organosulfur assimilation pathways in *E. coli*. The positive regulator protein CysB interacts with the co-inducer *N*-acetylserine to activate transcription of the *cys* biosynthetic operons [5] and the *cbl* gene [298]. Thiosulfate or sulfide act as anti-inducers. CysB also binds to the *tau* and *ssu* operators; it is required for *tau* activation [297], but not for transcription of *ssu* [222]. The Cbl protein acts as a positive regulator of the *tau* and *ssu* operons [297].

as sulfate-repressed *lacZ* fusions [218] or, because synthesis of the encoded proteins was determined by 2D-PAGE to be repressed in the presence of sulfate [22]. Expression of both the *tau* and the *ssu* operons requires the CysB protein [222,297], and these operons are therefore new members of the *cys* regulon. However, it was interesting that repression of the *tau* genes in the presence of sulfate was much more stringent than that observed for genes of the cysteine biosynthetic pathway [297]. This phenomenon was partly explained when it was discovered that expression of these genes requires not only CysB, but also a second, closely related regulator protein, the 'CysB-like' protein, Cbl, [297]. Like CysB, the Cbl protein is a member of the LysR family of transcriptional activators, and exhibits 41% amino acid identity to the CysB protein of *E. coli*. Expression of *cbl* is itself positively regulated by CysB, and CysB was shown to bind to the promoter region of the *cbl* gene [298]. Cbl also appears to be involved in the synthesis of other sulfate starvation-induced proteins, including the sulfate binding protein Sbp, and the acetylserine (thiol) lyase CysK [297]. Interestingly, *E. coli* contains second copies of both of these enzymes (CysP and CysM, respectively), and so the additional regulation by Cbl may represent a strategy to allow fine tuning of the cellular response to sulfate starvation.

More detailed studies of the role of Cbl have been carried out with the *tau* and *ssu* promoters. Cbl was found to bind upstream of the −35 region of both these promoters, and since expression of *tauA::lacZ* and *ssuE::lacZ* fusion required the presence of an intact *cbl* gene, it seems likely that Cbl is acting as a transcriptional activator for both these operons [222,297]. CysB also bound to both promoters, and binding was stimulated by *O*-acetylserine and by thiosulfate. At the *tau* promoter, CysB may well be acting as a co-activator, either binding together with Cbl, or changing the binding of Cbl in such as way as to stimulate transcriptional activation. For *ssu*, the situation is different. Band-shift studies suggested that the CysB binding site at the *ssu* promoter lies close to the transcription start site, and CysB may therefore act as a repressor. Although a *ssuE::lacZ* fusion was not expressed in a *cysB* mutant, expression of the *cbl* gene behind a *trc* promoter restored *ssuE* expression [222]. This showed that the requirement for CysB is indirect (*cbl* expression is itself part of the *cys* regulon, and therefore under CysB control).

Unlike for CysB, Cbl binding to *tau* or *ssu* promoter fragments was not stimulated by the addition of acetylserine. Sulfate did not enhance binding of either protein to the *tau* promoter, and thiosulfate did not stimulate binding to the *ssu* promoter region (in fact, the Cbl/*ssu* interaction was slightly weaker in the presence of 5 mM thiosulfate). The identity of the co-regulator of Cbl binding is therefore still unclear. As discussed above, structural studies of *K. pneumoniae* CysB have revealed the presence of a sulfate or acetylserine binding pocket in that protein [295], and many of the residues required for the interaction of CysB with inducer or anti-inducer are conserved in Cbl [222]. It therefore seems possible that the putative co-regulators of Cbl binding are also low-molecular-mass anions, but this remains to be demonstrated.

Control of sulfate assimilation by CysB appears to be very similar in *E. coli* and *S. typhimurium* [5,299]. It is therefore of interest that the *cbl* gene appears to be absent in *S. typhimurium* [297], and that this organism is also not able to grow with alkanesulfonates as sulfur source. In *K. pneumoniae*, by contrast, both the *cysB* and *cbl* genes are present, though the Cbl protein has not yet been characterized at a biochemical level. The CysB and Cbl proteins of *K. pneumoniae* are very closely related to their *E. coli* homologues (93% and 86% identity, respectively). *K. pneumoniae* is able to grow with taurine and other alkanesulfonates as sulfur source (unpublished), and presumably CysB and Cbl also play a role in controlling sulfonate-sulfur assimilation in this species.

The role of CysB and Cbl in the expression of the arylsulfatase operon ($atsBA_{Kp}$) in *K. pneumoniae* has not yet been established. Expression of the $atsBA_{Kp}$ operon is subject to complex regulation as part of the *moa* regulon in *K. pneumoniae* [135], but in the absence of tyramine or other related compounds its expression is repressed independently by sulfate or by cysteine [100]. This regulatory pattern is atypical for a purely CysB-controlled gene, but may correspond to combined CysB/Cbl control, as seen for taurine metabolism in *E. coli*.

In pseudomonads the situation is a little different. In *P. aeruginosa* cysteine biosynthesis differs from the well-characterized pathway of *E. coli* in that sulfate activation is catalyzed by only two proteins (CysDN), rather than three (CysDNC), and that sulfide is transferred to *O*-succinylhomoserine (rather than *O*-acetylserine), giving homocysteine as the initial organosulfur product (Fig. 1) [19]. Cysteine is then generated by reverse transsulfurylation via cystathionine [148]. The presence of the reverse transsulfurylation pathway allows *P. aeruginosa* to grow readily with methionine as sole sulfur source, in contrast to *E. coli*, which grows only very slowly under these conditions. A *cysB* mutant of *P. aeruginosa* can grow with sulfite or with methionine as sole sulfur sources, demonstrating that sulfite reductase and the reverse transsulfurylation pathway from methionine to cysteine are independent of CysB [14]. However, a *cysB* mutant was unable to utilize either sulfate esters or sulfonates. Expression studies with the *atsR* and *atsBCA* genes of *P. aeruginosa* confirmed that expression of the *ats* genes required an intact *cysB* gene. Analysis of the *P. aeruginosa* genome sequence shows only one gene that resembles *E. coli cysB*, and so there appears to be no equivalent to the *cbl* gene that is found in enteric bacteria.

However, an additional regulatory factor(s) must nonetheless be present. Arylsulfatase synthesis in *P. aeruginosa* is repressed independently by sulfite and sulfide, but not by sulfate [20], whereas methanesulfonatase synthesis is

repressed by all three compounds independently [14] (Fig. 14). Sequence analysis of the *ats* and *msu* promoter regions did not provide any evidence for a CysB binding site, though it should be stressed that the consensus for these sites is weak. It will therefore be interesting to see whether future binding studies confirm a direct role for CysB in regulating these genes, or lead to the conclusion that CysB-mediated regulation is also indirect, involving additional, as yet unidentified factors. Additional factors could provide the means for adjusting the response to sulfate limitation, as seen for the Cbl protein in *E. coli*.

In some cases the sulfate starvation response is also modulated by a positive regulator that responds not only to sulfate limitation but to the presence of specific alternative sulfur sources. Regulation of *asfABC* by AsfR, for instance, is positively affected by toluenesulfonate or benzenesulfonate, and repressed in the presence of sulfate – this repression appears to be partly CysB-mediated [185]. AsfR is related (34% identity) to SdsB, the positive regulator protein involved in dodecylsulfate utilization in *Pseudomonas* sp. ATCC 19151 [166], and a further protein of the same family (45% identity to AsfR) is encoded downstream of *atsR* in *P. putida* (unpublished). It is not yet known how these latter two genes are controlled, but their close relatedness to AsfR and CysB makes it likely that they are involved in regulating some aspect of organosulfur metabolism.

Organosulfur metabolism in fungi and cyanobacteria is regulated by other mechanisms. In the filamentous fungus *Neurospora crassa*, sulfatase expression is positively regulated by the CYS3 protein, a bZIP DNA binding protein that is subject to positive autoregulation, and controls expression of genes encoding cysteine biosynthesis, sulfate permeases, cholinesulfatase and arylsulfatase [300,301]. The *cys3* gene is subject to negative regulation by two further proteins, the products of the *scon1* and *scon2* genes. The CYS3 protein is also subject to rapid turnover in response to changes in sulfur supply [302]. Related control mechanisms are also present in the yeast *S. cerevisiae* [9,303]. In the cyanobacterium *Synechococcus* sp. strain PCC7942 a further type of regulator is involved. The CysR protein, a protein of the Crp family, has been implicated in the expression of several plasmid-encoded genes of sulfur metabolism [304,305]. The green alga *Chlamydomonas reinhardtii* requires the SAC1 protein for survival during sulfur deprived conditions, and for expression of the arylsulfatase gene [306]. SAC1 appears to be an integral membrane protein, and the regulatory effect may therefore be due to import of an essential regulatory molecule. A related gene is also found in the cyanobacterium *Synechocystis* [142], and it will be interesting to see whether it also plays a role in controlling the cellular response to sulfur deprivation.

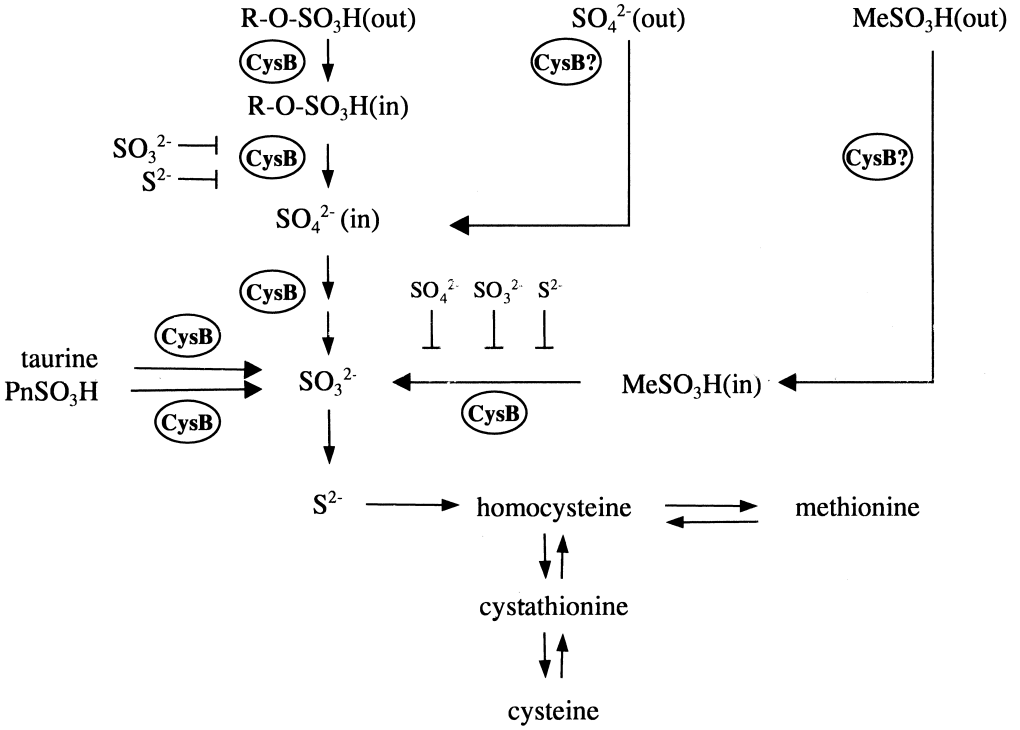


Fig. 14. Regulation of organosulfur assimilation pathways in *P. aeruginosa*. Growth of *P. aeruginosa* with sulfate, sulfonates or sulfate esters requires the CysB protein. Expression of arylsulfate transport and of arylsulfatase requires CysB and the latter is repressed by sulfite or sulfide, but not sulfate. Methanesulfonatase synthesis requires CysB and is repressed independently by sulfate, sulfite or sulfide. CysB is not required for growth with methionine or sulfite.

9. Uptake of sulfonates and sulfate esters

Even a superficial inspection of the list of sulfate starvation-induced proteins identified in 2D-PAGE studies (Table 1) reveals that synthesis of several periplasmic solute binding proteins is regulated by sulfur supply. This result may in part reflect the soluble nature of these proteins, and the fact that they are readily extracted from the cell and visualized by 2D-PAGE, but it also suggests that induction of transport systems is an important part of the bacterial cell's response to sulfate limitation. This hypothesis is confirmed by the fact that the transcriptional units encoding the sulfatase or sulfonatase genes themselves often also contain genes for putative ABC-type transport systems (Figs. 6, 8 and 10). Hence, the *tauD* gene of *E. coli* is preceded by *tauABC* [297], and the *ssuD* gene of *B. subtilis* is located in the same operon as *ssuABC* [223], as is also the case for the *ssuD* genes of both *P. putida* and *P. aeruginosa*. The three loci that are required for arylsulfonate desulfonation in *P. putida* (*ssu, ats* and *asf*, Fig. 10 [185]) all contain genes which appear to encode components of ABC-type transporters. From mutant studies, the AtsRBC system catalyzes sulfate ester transport in *P. aeruginosa* and *P. putida* [131,136], and its expression is also repressed during growth with sulfate. Most of the intracellular (Cys-type) sulfatase genes in Fig. 6 are also associated with transport systems of some nature, whereas the periplasmic (Ser-type) homologues are not. The requirement for sulfate ester and sulfonate transport systems is not surprising, since these compounds are completely ionized at physiological pH, and active transport systems are required to allow them to enter the cell.

When the known periplasmic binding proteins for sulfonates and sulfate esters are compared (Fig. 15) considerable similarity is found among them [24]. These proteins form an independent family of binding proteins separate from those previously defined by Tam and Saier [307], being 22–45% identical to each other, but sharing less than 15% sequence identity with the families of solute binding proteins that have previously been described. Surprisingly, they are also only moderately related to Sbp and CysP, the periplasmic sulfate and thiosulfate binding proteins, respectively. These two proteins are closely related to each other and show an overlapping substrate specificity [308].

To date, the only detailed biochemical study of arylsulfonate transport into the cell reported an inducible secondary proton symport system for uptake of toluenesulfonate by *Comamonas testosteroni* T-2 [309]. This study was complicated by the fact that the strain uses toluenesulfonate as a carbon and energy source, and that the sulfonate was therefore metabolized further immediately after entry into the cell. The genes encoding the inducible desulfonation system for toluenesulfonate in this strain have recently been reported (*tsaMBCD*/*tsaR*) [310], but the transport system was not encoded in the same locus, and one can

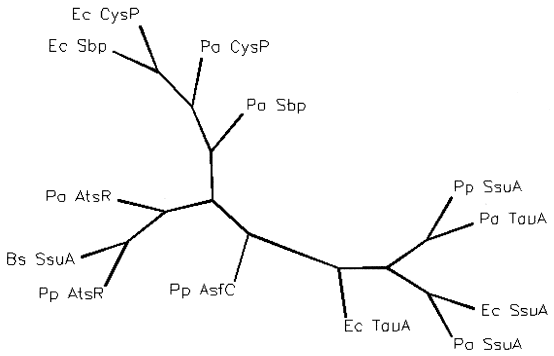


Fig. 15. Phylogenetic tree for sulfate, thiosulfate, sulfonate and sulfate ester binding proteins. The proteins shown are the putative sulfonate binding protein SsuA from *B. subtilis* (Bs), *P. aeruginosa* (Pa), *E. coli* (Ec), and *P. putida* (Pp), the sulfate ester binding protein AtsR from *P. putida* and *P. aeruginosa*, the taurine binding protein TauA from *E. coli* and *P. aeruginosa*, the putative arylsulfonate binding protein AsfC from *P. putida*, and the sulfate and thiosulfate binding proteins Sbp and CysP from *E. coli* and *P. aeruginosa*. The mature protein sequences (after removal of the signal peptides) were aligned using CLUSTALW, and the phylogenetic tree produced with DRAWTREE.

therefore only speculate on its relationship to the sulfate-regulated transport systems described above.

Specific permeases that catalyze the uptake of several different sulfate esters have also been identified in *N. crassa* [31]. Synthesis of these proteins is controlled by the CYS3 protein and repressed during growth with preferred sulfur sources. Sulfate-repressed uptake of alkanesulfonic acids has also been observed in algae [311,312], and in cyanobacteria [312,313], but the regulatory mechanism has not yet been explored in these species.

10. Organosulfur utilization without oxygen

Sulfonates and sulfate esters in soils are present not only in aerobic niches, but are also found in deeper, anaerobic soils [45]. A range of anaerobic bacteria have been identified which can make use of sulfonates. Most of these isolates belong to novel genera, and this uncharted biodiversity is just starting to be explored [7].

Under anaerobic conditions, sulfonates and sulfate esters can in principle provide not only a source of assimilable sulfur but can also be used in dissimilatory pathways (reviewed recently by Cook et al. [7] and by Lie et al. [8]). The first anaerobes reported to assimilate sulfonate sulfur were *C. pasteurianum* C1, which desulfurizes isethionate, taurine, or toluenesulfonate [314] and *Clostridium beijerincki* EV4, which grows with toluenesulfonate as sulfur source [315]. The mechanism by which this aromatic desulfonation takes place is still unclear, but is clearly different from the desulfonative process catalyzed by *P. putida.* NMR and LC-MS analyses of the isolated desulfonation products suggested that the aromatic ring may undergo further substitution before desulfonation occurs (unpublished results). Further enrichments for isolates that can

assimilate toluenesulfonate sulfur have also yielded *Clostridium* species [316], and it is possible that this ability is limited to this genus. By contrast, a facultatively anaerobic *Klebsiella* isolate was able to utilize cysteate sulfur under fermentative conditions, but could not desulfurize taurine, isethionate or toluenesulfonate [314], and a novel anaerobic member of the γ-proteobacteria related to *Shewanella* and *Aeromonas*, strain RZLAS, was able to grow with LAS as sole sulfur source [317].

Sulfonates can also serve either as electron donors or electron acceptors in respiration, or as substrate in disproportionation reactions under fermentative conditions. Various sulfate-reducing bacteria use isethionate, cysteate or taurine as electron acceptor [8,318,319]. The taurine desulfonation reaction is catalyzed by transamination to sulfoacetaldehyde and subsequent desulfonative hydrolysis to acetate, formally the same mechanism as used by pseudomonads during growth with taurine as a carbon source [200,203,213]. Taurine desulfonation as electron acceptor was also found in a sulfate non-reducer, the Gram-negative anaerobe *Bilophila wadsworthia* RZATAU [318]. In this organism the enzymes of taurine transformation were reported to be constitutively expressed [7] (by contrast, in *Desulfovibrio* the taurine aminotransferase is inducible [319]). The sulfite released by *B. wadsworthia* sulfoacetaldehyde hydrolase may be reduced to sulfide by desulfoviridin, which was detected in extracts of this strain [7].

Sulfonates are also used as electron donors by an *Alcaligenes* strain [205] and a *Paracoccus denitrificans* isolate [320], in the presence of nitrate as electron acceptor. In *Alcaligenes* sp. strain NKNTAU taurine was desulfonated to sulfite, which was quantitatively oxidized to sulfate [205], and similar results were obtained with isethionate and cysteate in other species [7]. A novel, obligately anaerobic Gram-negative isolate, named *Desulforhopalus singaporensis*, could ferment taurine, but not other sulfonates, to sulfide [321]. Two Gram-positive species have also been identified that are able to ferment cysteate or taurine. Taurine was converted by strain GKNTAU (which belongs to a novel genus within the *Clostridium* group [7]) to thiosulfate, presumably via transamination to sulfoacetaldehyde [207]. A *Desulfovibrio* isolate was also able to ferment cysteate to equimolar amounts of sulfite and sulfide [322].

In contrast to the extensive recent work on the anaerobic utilization of sulfonates, very little is known about how sulfate esters are degraded under anaerobic conditions. Clostridia were able to grow with nitrocatechol sulfate or 5-bromo-4-chloro-3-indolylsulfate, but the corresponding phenol products were not detected, suggesting that sulfate ester utilization in these species is catalyzed by a fundamentally different enzyme from the arylsulfatases that have been characterized in Gram-negative, aerobic bacteria [316]. Further studies are required to determine the nature of sulfate ester utilization in clostridia, and biodiversity studies on the distribution of Cys- and Ser-type sulfatases in aerobic and anaerobic niches are also still outstanding.

11. Conclusions and perspectives

The purpose of this article has been to present an overview of recent advances in our understanding of how Gram-negative bacteria contribute to the global sulfur cycle by their metabolism of organosulfur compounds such as sulfate esters and sulfonates. By concentrating on enzymes and enzyme systems whose synthesis is controlled by the sulfur supply to the cell, it has emphasized a new aspect which has become particularly relevant in the last few years. Sulfate esters and sulfonates can be used as sulfur sources for growth by a variety of bacterial species, but the number of organisms that can carry out complete mineralization of these compounds is more limited. The substrate specificity of enzymes that are induced as part of a sulfate-starvation response is often quite broad, allowing the cell considerable flexibility in scavenging sulfur from the environment. Whereas this characteristic has obvious advantages in designing practical applications for such enzyme systems, there are also grave disadvantages – enzymes that are used to provide the organism with sulfur are usually present only in low amounts in the cell, and their synthesis is switched off in the presence of better sulfur sources such as sulfate or sulfite.

The understanding of sulfur-regulated systems is, however, providing means to avoid these drawbacks. Thus, the first steps have been taken towards practical coal and oil biodesulfurization using the dibenzothiophene desulfurization genes by removing the sulfate control of their expression, and starting to optimize a desulfurization strain [286]. Similar work is still required for strains that desulfurize aromatic sulfonates. Whereas many such sulfonates are readily biodegradable, others are persistent and appear in drinking water after bank filtration. Most of the genetic understanding of aromatic desulfonation processes as part of the sulfur cycle comes from *P. putida*, and it will be important to evaluate how far this knowledge is applicable to other organisms, and whether the genes identified are relevant in biodegradation under non-laboratory conditions.

This last question is also crucial for investigating a further outstanding aspect of organosulfur biochemistry, the question of soil organosulfur cycling. With the characterization of sulfonate and sulfate ester metabolic systems we now have first tools in hand for the elucidation of the molecular processes that occur in the microbially mediated cycling of sulfur between inorganic and organically bound forms in the soil. Practical applications can be readily visualized, with the aim of increasing the levels of plant-accessible sulfate in the soil, and increasing crop yields on sulfur-deficient soils. Such applications might involve

field inoculation with genetically modified organisms, but it is perhaps more realistic to think of monitoring bacterial genetic markers in order suitably to modify fertilization regimes and thereby optimize release of soil sulfur for plant growth by indigenous bacteria. These genes also show promise as bio-indicators for available sulfur in the soil, but more work needs to be done to characterize their expression in native environments.

Regulation of the sulfonatase and sulfatase genes in Gram-negative bacteria has been found to be ultimately controlled by the CysB protein, which acts as a global regulator in all of sulfur metabolism. However, much remains to be learned about the fine tuning of this control. In at least two cases, additional regulatory proteins are required for sulfonatase gene expression (Cbl in enteric bacteria [297], and AsfR in *P. putida* [185]), and it is to be anticipated that more will be discovered, especially resulting from the new data that are appearing from whole genome analysis. Elucidating and manipulating the mechanisms by which these regulatory cascades function will provide interesting challenges for the coming years.

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