Sulfatases are a highly conserved family of enzymes found in all three domains of life. To be active, sulfatases undergo a unique post-translational modification leading to the conversion of either a critical cysteine (“Cys-type” sulfatases) or a serine (“Ser-type” sulfatases) into a Ω-formylglycine (FGly). This conversion depends on a strictly conserved sequence called “sulfatase signature” (C/S)XPXR. In a search for new enzymes from the human microbiota, we identified the first sulfatase from Firmicutes. Matrix-assisted laser desorption ionization time-of-flight analysis revealed that this enzyme undergoes conversion of its critical cysteine residue into FGly, even though it has a modified (C/S)XPXR sulfatase signature. Examination of the bacterial and archaeal genomes sequenced to date has identified many genes bearing this new motif, suggesting that the definition of the sulfatase signature should be expanded. Furthermore, we have also identified a new Cys-type sulfatase-maturing enzyme that catalyzes the conversion of cysteine into FGly, in anaerobic conditions, whereas the only enzyme reported so far to be able to catalyze this reaction is oxygen-dependent. The new enzyme belongs to the radical S-adenosyl- L-methionine enzyme superfamily and is related to the Ser-type sulfatase-maturing enzymes. This finding leads to the definition of a new enzyme family of sulfatase-maturing enzymes that we have named anSME (anaerobic sulfatase-maturing enzyme). This family includes enzymes able to mature Cys-type as well as Ser-type sulfatases in anaerobic conditions. In conclusion, our results lead to a new scheme for the biochemistry of sulfatases maturation and suggest that the number of genes and bacterial species encoding sulfatase enzymes is currently underestimated.

Sulfatases are widespread enzymes found from prokaryotes to eukaryotes. They are involved in various metabolic processes, ranging from sulfate starvation response in bacteria to hormone biosynthesis and the modulation of developmental cell signaling in mammals (1). In humans, their biological relevance is particularly underlined by their involvement in several inherited diseases such as mucopolysaccharidoses (2), metachromatic leukodystrophy (3), X-linked ichthyosis, chondrodysplasia punctata (4), and the rare multiple sulfatase deficiency syndrome (1, 5).

Sulfatases act on a broad diversity of substrates, which leads to their classification by the IUBMB into 17 classes (from EC 3.1.6.1 to EC 3.1.6.18). Despite this apparent heterogeneity, the primary and tertiary structures of sulfatases are highly conserved (6–9). Probably, the most striking feature of sulfatases is that they undergo a unique co- or post-translational modification that produces a Ω-formylglycine (FGly)2 residue in their active site (10, 11). This residue originates from the conversion of a serine (in prokaryotes) or a cysteine (in prokaryotes and eukaryotes), thus defining two classes of sulfatases, the “Ser-type” and the “Cys-type” sulfatases (10, 11).

These unique modifications are mediated by one of two different enzymes, formylglycine-generating enzyme (FGE) (12, 13) and AtsB (14), responsible for the conversion of cysteine or serine to FGly, respectively. Both systems are highly divergent but recognize the same consensus motif “(C/S)XPXR” regarded as the “sulfatase signature” (15–17). This consensus sequence is conserved across all known members of the sulfatase family and has been described as being essential for the conversion of cysteine or serine to FGly and to the proper conformation of the active site of sulfatases (15–17).

In a search for enzymes from human microbiota that are able to hydrolyze nutritionally relevant sulfated compounds, namely glucosinolates (β-thioglucoside-N-hydroxysulfates) (18), we investigated the biochemical activities of Clostridium perfringens. This led us to identify the first sulfatase from Firmicutes. This sulfatase, a Cys-type, is the first one described that lacks the canonical sulfatase signature sequence. Furthermore, while searching for enzymes responsible for its maturation in vivo, we could not identify any FGE-type enzyme, the only Cys-type maturing enzyme identified to date. Instead, we discovered the C. perfringens genome encodes a protein related to the Ser-type sulfatase-maturing enzyme, AtsB. We demonstrate that this enzyme activates the newly discovered sulfatase, making it the first enzyme described as being able to convert cysteine to FGly in anaerobic bacteria.

EXPERIMENTAL PROCEDURES

Chemicals—p-Nitrophenyl sulfate was purchased from Sigma. Enzymes, oligonucleotides, and culture media were pur-

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chased from VWR Scientific. Other chemicals and reagents were obtained from commercial sources and were of analytical grade.

**Bacterial Strains, Plasmids, and DNA Manipulations**—The *C. perfringens* strain used in this study was the ATCC 13124 strain. *Escherichia coli* DH5α was used for routine DNA manipulations. *E. coli* BL21 (DE3) (Stratagene) was used for *C. perfringens* sulfatase and maturation enzyme overexpression. The pET-28(a) plasmid (Novagen) used to clone the *cpe0231* gene was engineered to bear the ampicillin resistance gene. The *cpe0635* gene was cloned in the pRSF plasmid (Novagen). T4 DNA ligase was from Promega. The plasmid DNA purification kit and QIAprep spin were from Qiagen. DNA fragments were extracted from agarose gel and purified with Wizard SV gel and PCR clean up system kit (Promega). DNA sequencing was performed by VWR Scientific.

**Preparation of *C. perfringens* Protein Extracts**—*C. perfringens* ATCC 13124 was grown overnight in an anaerobic chamber (Bactron, Cornelius, OR) in TYH broth (tryptone, yeast extract, and hemin broth) supplemented with or without 50 mM ammonium sulfate. At the end of the culture, the cells were harvested by centrifugation (4,000 × g at 4 °C for 20 min) and disrupted by sonication (1 min). After centrifugation (10,000 × g at 4 °C for 20 min), the resulting supernatant constituting the protein extract was stored at −20 °C.

**Enzyme Assays**—Sulfatase activity was assayed at 30 °C for 10 min using 50 mM p-nitrophenyl sulfate in 100 mM Tris buffer, pH 7.15. The p-nitrophenol released was measured spectrophotometrically at 405 nm (ε = 9,000 mol⁻¹·cm⁻¹ at pH 7.15).

**Cloning and Construction of the pET-6His-CPE0231 Overexpressing Plasmid**—*C. perfringens* ATCC 13124 was grown anaerobically in BHI medium, pH 7.0, and the cells were harvested to extract the genomic DNA using the Wizard genomic DNA purification kit from Promega. The *cpe0231* gene encoding the putative sulfatase was amplified by PCR-based method using genomic DNA obtained from commercial sources and were of analytical quality. E. coli pET-28(a) plasmid (Novagen) used to clone the *cpe0231* gene was cloned in the pRSF plasmid (Novagen). T4 DNA ligase was from Promega. The plasmid DNA purification kit and QIAprep spin were from Qiagen. DNA fragments were extracted from agarose gel and purified with Wizard SV gel and PCR clean up system kit (Promega). DNA sequencing was performed by VWR Scientific.

**MALDI-TOF Analysis**—Samples were prepared as follows. The overexpressed protein (50 µmol·µl⁻¹) was digested overnight with trypsin (20 ng·µl⁻¹) in ammonium carbonate buffer, pH 8.0, at 37 °C. Then 5 µl of the solution was further hydrolyzed with 5 µl of CNBr (20 mg·mL⁻¹ in 0.2 M HCl) in the dark at 45 °C for 4 h.

The α-cyano-4-hydroxycinnamic acid matrix (CHCA) was prepared at 4 mg·mL⁻¹ in 0.15% trifluoroacetic acid, 50% acetonitrile. The 2,4-dinitrophenyldrazonized acid matrix (DNPH) was prepared at 1.3 mg·mL⁻¹ in 0.5% trifluoroacetic acid, 50% acetonitrile. Equal volumes (1 µl) of matrix and sample were spotted onto the MALDI-TOF target plate. MALDI-TOF analysis was then performed on a Voyager DE STR Instrument (Applied Biosystems, Framingham, CA). Spectra were acquired in the reflector mode with 20-kV accelerating voltage, 62% grid voltage, and a 120-ns delay.

**RESULTS**

**Sulfatase Activity of *C. perfringens***—Protein extract from *C. perfringens* was assayed for sulfatase activity using the...
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**Species (Gene) | Sequence**
---|---
Pa (Apta) | LAIALLLDDFHTA-51TEGRYNF
Kp (Apta) | MABGMLMQYTTY-51TEGRYNF
Cr (Apta) | INVYVLYQTVITV-51TEGRYNF
Vc (Q10723) | IRYVFMELKLYNVTVP-51TEGRYNF
Nc (Apsl) | LABETFTYHYCTTA-51TEGRYNF
Sp (P50473) | MANPOLRFGTQ decorating 51-71 peptide
Hp (Ars) | MARLNSFTPQVVGD1-51TEGRYNF
Gg (Ars) | LABLWRLTQHTRAPILAPEPFAFLRPFY
Mm (Ars) | LABALFLRRFTFPVQFLRPAFLRPFY
Ns (Ars) | LAAPQLTFTTQVQFLRPAFLRPFY
Ns (Ars) | LAAPQLTFTTQVQFLRPAFLRPFY

**Sulfatase signature**

**CPE0231**

— Sequence alignment of several authentic sulfatases representative of bacteria (Pa, Pseudomonas aeruginosa (36); Kp, K. pneumoniae (14)), protists (Cr, Chlamydomonas reinhardtii (38); Vc, Volvox carteri (39)), fungi (Nc, Neurospora crassa (40)), lower eukaryotes (Sp, Strongylocentrotus purpuratus (41); Hp, Hemicentrotus pulcherrimus (42)), birds (Gg, Gallus gallus (43)), mammals (Mm, Mus musculus (44); Hs, Homo sapiens (45, 46)), and the cloned sulfatase from C. perfringens (CPE0231).

Gene names or the protein accession numbers, when no name is available, are given in parentheses.

synthetic substrate, p-nitrophenyl sulfate (PNP-S). At neutral pH, the extract exhibited a weak sulfatase activity of 0.25 nmol·min⁻¹·mg⁻¹, which almost vanished when we grew C. perfringens in presence of 50 mM ammonium sulfate. This result suggested that C. perfringens possesses at least one sulfatase enzyme regulated by the sulfate content of the medium.

A search for genes annotated as sulfatases in the C. perfringens genome did not show any obvious candidates (15), whereas a blast search with sulfatase sequences led to the identification of one gene, cpe0231, annotated as a “probable phosphonate monoester hydrolase” (21, 22). Despite its annotation, the sequence of the putative protein coded by the cpe0231 gene shared significant identity (from 20 to 24%) with other known bacterial sulfatases. Nevertheless, although all the sequenced sulfatases studied to date share a consensus motif (C/S)X(C/R), i.e. a cysteinyl residue involved in sulfate hydrolysis and thus to undergo the conversion into FGly. To provide evidence of this conversion, we applied MALDI-TOF analysis to the peptides released from a tryptic/CNBr digestion. We clearly obtained two relevant peptides, labeled Cys-51–71 and FGly-51–71 (Fig. 2A). The former has a molecular mass of 2263.96 Da and thus corresponds to the peptide 51–71 ATEGYNFENAYTAVPSCIASR 71 (theoretical molecular mass of 2264.03 Da) containing the critical cysteine Cys-67; the latter has a molecular mass of 2246.03 Da, i.e. 18 Da less, as expected from the conversion of the cysteine residue into FGly (Fig. 2A). To ascertain the nature of the modification undergone by the peptide 51–71, we performed MALDI-TOF analysis using DNP as a matrix. Indeed, FGly-containing peptides specifically react with DNP to form a hydrazone derivative with a mass increment of 180 Da (23). With the DNP hydroxylamine derivatization, we were able to detect a large amount of the nonmaturated peptide Cys-51–71 (Fig. 2).

Thus, the protein encoded by the cpe0231 gene, as with all the sulfatases studied to date, is endowed with a conversion of its critical residue into FGly. However, this sulfatase possesses the sequence “FGlyXAXXR” at its active site, whereas the other known sulfatases, regardless of their origin (i.e. prokaryotic or

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**FIGURE 1.** Sequence alignment of several authentic sulfatases representative of bacteria (Pa, Pseudomonas aeruginosa (36); Kp, K. pneumoniae (14)), protists (Cr, Chlamydomonas reinhardtii (38); Vc, Volvox carteri (39)), fungi (Nc, Neurospora crassa (40)), lower eukaryotes (Sp, Strongylocentrotus purpuratus (41); Hp, Hemicentrotus pulcherrimus (42)), birds (Gg, Gallus gallus (43)), mammals (Mm, Mus musculus (44); Hs, Homo sapiens (45, 46)), and the cloned sulfatase from C. perfringens (CPE0231).

Gene names or the protein accession numbers, when no name is available, are given in parentheses.

| Species (Gene) | Sequence |
|---|---|
| Pa (Apta) | LAIALLLDDFHTA-51TEGRYNF |
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| Sp (P50473) | MANPOLRFGTQ decorating 51-71 peptide |
| Hp (Ars) | MARLNSFTPQVVGD1-51TEGRYNF |
| Gg (Ars) | LABLWRLTQHTRAPILAPEPFAFLRPFY |
| Mm (Ars) | LABALFLRRFTFPVQFLRPAFLRPFY |
| Ns (Ars) | LAAPQLTFTTQVQFLRPAFLRPFY |
| Ns (Ars) | LAAPQLTFTTQVQFLRPAFLRPFY |

**Sulfatase signature**

CPE0231

— Sequence analysis of the protein encoded by the cpe0231 gene indicated that Cys-51 (Cys-67 with the addition of the Tag in the recombinant enzyme) is likely to be the key residue involved in sulfate hydrolysis and thus to undergo the conversion into FGly. To provide evidence of this conversion, we applied MALDI-TOF analysis to the peptides released from a tryptic/CNBr digestion. We clearly obtained two relevant peptides, labeled Cys-51–71 and FGly-51–71 (Fig. 2A). The former has a molecular mass of 2263.96 Da and thus corresponds to the peptide 51–71 ATEGYNFENAYTAVPSCIASR 71 (theoretical molecular mass of 2264.03 Da) containing the critical cysteine Cys-67; the latter has a molecular mass of 2246.03 Da, i.e. 18 Da less, as expected from the conversion of the cysteine residue into FGly (Fig. 2A). To ascertain the nature of the modification undergone by the peptide 51–71, we performed MALDI-TOF analysis using DNP as a matrix. Indeed, FGly-containing peptides specifically react with DNP to form a hydrazone derivative with a mass increment of 180 Da (23). With the DNP matrix a new peptide with the expected molecular mass of 2425.95 Da appeared (Fig. 2B). Nevertheless, in E. coli, the overexpressed sulfatase was only partially maturated, as we were able to detect a large amount of the nonmaturated peptide Cys-51–71 (Fig. 2).
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Planctomycetes (Rhodopirellula), Cyanobacteria (Crocosphaera), Bacteroidetes (Bacteroides), and in two new phyla regarded until recently as deprived of sulfatases (17). These phyla are the large phylum of the Firmicutes and the recently described phylum of the Acidobacteria (25). We also identified potential sulfatases with the CXAXR motif in one archaeal genus, Methanosarcina.

Examination of the genomes encoding sulfatases with the CXAXR motif revealed various situations. Some genomes, as those of C. perfringens or Methanosarcina mazei, only encode sulfatases with the CXAXR motif, whereas others, as those of Rhodopirellula baltica or Solibacter usitatus, encode both types of cysteine sulfatases, i.e. with either the “CXPXR” or the CXAXR motif. Finally, we also found, although less frequently, genomes encoding potential serine sulfatases with an “SXAXR” motif, as exemplified by Vibrio parahemolyticus, Vibrio vulnificus, and Bacteroides thetaiotaomicron.

DISCUSSION

In a search for enzymes from C. perfringens able to hydrolyze sulfated compounds, namely glucosinolates (18, 26), we found that a protein extract of C. perfringens exhibits a sulfatase activity toward the synthetic substrate PNP-S.

eukaryotic) or their type (Ser-type or Cys-type), bear the sequence FGlyXPXR.

Cloning and Sulfatase Maturation Activity of the cpe0635 Gene—C. perfringens does not encode any FGE-type enzyme, the only Cys-type maturation enzyme identified to date. Nevertheless, an examination of C. perfringens genome revealed the existence of the gene cpe0635, located almost 500 kbp downstream of the sulfatase gene. This gene codes for a protein that shares homology (48%) with AtsB, the maturation enzyme of Ser-type sulfatases in Klebsiella pneumoniae (14). AtsB has been described to be strictly dependent on the presence of an N-terminal signal peptide and on a critical serine residue in the sulfatase active site (24), both features being absent with C. perfringens sulfatase.

We cloned CPE0635 into a plasmid allowing co-expression with the C. perfringens sulfatase gene in E. coli BL21 (DE3). As shown in Fig. 3 (lane B), we obtain an efficient co-expression of both proteins. As only the sulfatase bears a His tag, it was easily purified and further analyzed to check if co-expression with the cpe0635 gene enhanced the sulfatase maturation.

MALDI-TOF analysis revealed that when co-expressed with the cpe0635 gene, the sulfatase was fully mature because no immature Cys-51–71 peptide could be detected (Fig. 4A). Furthermore, analysis with the DNPH matrix confirmed the nature and the efficiency of the maturation, as the signal originating from the peptide “FGly-DNP” dominates the MALDI-TOF spectrum (Fig. 4B). As a consequence of a more efficient maturation, the specific activity of C. perfringens sulfatase increased more than 6-fold, rising from 0.08 to 0.53 μmol·min⁻¹·mg⁻¹.

“CXAXR” Sulfatases in Other Bacteria—We searched to see if the C. perfringens sulfatase motif CXAXR exists in other potential microbial sulfatases. For this purpose we performed a blast search, using the following motif (CS)XAXR, in all the bacterial genomes sequenced thus far. Only sequences that have significant homologies to authentic sulfatases were further considered. We found several genes encoding potential sulfatases lacking the consensus proline at position 3 of the sulfatase signature. These genes were found in 22 bacterial species corresponding to the major bacterial phyla (Fig. 5): Proteobacteria (Bordetella, Bradyrhizobium, Colwellia, Mesorhizobium, Novosphingobium, Pseudoalteromonas, Pseudomonas, Ralstonia, Shewanella, Silicibacter, Sinorhizobium, and Vibrio),
Nevertheless, the purified protein exhibited sulfatase activity and shares many common features with sulfatases described previously. Its activity is increased by calcium, a co-factor found in most of the active sites of sulfatases studied thus far (6–9), and, above all, it undergoes the post-translational modification, unique to sulfatase enzymes (10, 15), namely the conversion of the cysteine residue present in the new motif CXXXR into a FGly. The sulfatase from C. perfringens is thus an authentic sulfatase bearing the motif FGlyXXXR in its active site and should be classified in the JUBMB subclass of arylsulfatases (EC 3.1.6.1).

These results rule out the current paradigm defining the sulfatase signature (17, 27) and demonstrate that the definition of the sulfatase signature should be expanded to include the novel motif CXXXR. Our results also indicate that the number of genes encoding sulfatases and of bacterial species bearing sulfatase enzymes is currently underestimated. This has biochemical significance because recent findings highlight the involvement of sulfatases in the pathogenic properties of bacteria (29, 30).

Our study also brings new data in the general scheme of prokaryote sulfatase maturation. Until now it was considered that the maturation of sulfatases is dependent on two enzymatic systems, according to the nature of the amino acid residue encoded in the active site. The first, which has attracted considerable attention, is FGE, an oxygen-dependent oxidoreductase found from prokaryotes to eukaryotes (12, 13, 31). This enzyme is the only one described to date able to convert cysteine into FGly (12, 28, 32, 33).

The other system, which accounts for the maturation of Ser-type sulfatases, is called AtsB and probably belongs to the radical S-adenosyl-L-methionine enzyme superfamily (34). AtsB has been reported previously to be specific of sulfatases described previously. Its activity is increased by calcium, a co-factor found in most of the active sites of sulfatases studied thus far (6–9), and, above all, it undergoes the post-translational modification, unique to sulfatase enzymes (10, 15), namely the conversion of the cysteine residue present in the new motif CXXXR into a FGly. The sulfatase from C. perfringens is thus an authentic sulfatase bearing the motif FGlyXXXR in its active site and should be classified in the JUBMB subclass of arylsulfatases (EC 3.1.6.1).

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The other system, which accounts for the maturation of Ser-type sulfatases, is called AtsB and probably belongs to the radical S-adenosyl-L-methionine enzyme superfamily (34). AtsB has been reported previously to be specific of sulfatases bearing the SXXXR motif and to be strictly dependent on the presence of a signal peptide (24). Furthermore, yeast two-hybrid assays have shown that the presence of cysteine instead of a serine in the active site disrupted AtsB/Sulfatase interactions (24, 35) preventing further maturation of the enzyme.
In *C. perfringens* we were unable to identify any FGE-related protein able to account for the physiological maturation of the cloned Cys-type sulfatase. Surprisingly, we identified a gene encoding a protein showing high homology with AtsB, the enzyme responsible for the maturation of Ser-type sulfatases. AtsB and the enzyme found in *C. perfringens* not only have high sequence identity but also bear the Cx,Cx,C motif, which is a signature for radical S-adenosyl-L-methionine enzymes (34).

Co-expression of both genes, encoding the sulfatase and the putative maturation enzyme, led to a fully maturated sulfatase. Thus, in *vivo*, *C. perfringens* sulfatase is not modified by an FGE-type enzyme but by an enzyme closer to the Ser-type sulfatase maturation machinery.

The Cys-type maturing enzyme identified in *C. perfringens* strongly differs from the other Cys-type maturing enzyme (FGE) because it is able to catalyze the conversion of cysteine into FGly under strict anaerobic conditions. Indeed, FGE has been reported recently to be strictly dependent on molecular oxygen (28, 33), which raised the question of how Cys-type sulfatases can be maturated in anaerobic or facultative aerobic bacteria?

The new maturation enzyme identified in *C. perfringens*, a strictly anaerobic microorganism, answers this question, at least in part, and provides evidence that Cys-type sulfatases can be maturated by oxygen-independent oxidoreductases. Supporting this conclusion, a search among sequenced bacterial and archaeal genomes showed that strict anaerobes never bear FGE-related genes but rather AtsB-related genes like many facultative anaerobes such as *E. coli*.

Until now it was unclear why *E. coli*, while being deprived of any FGE-related enzyme, is able to mature Cys-type sulfatases (36, 37). This led to the hypothesis of the existence of a yet unidentified system responsible for the maturation of Cys-type sulfatases among prokaryotes (11, 14).

Our findings, in agreement with the recent bio-computing study of Ballabio and co-workers (17), demonstrate that bacteria such as *C. perfringens*, which possess only enzymes related to AtsB, are able to mature Cys-type sulfatases. Thus, it is tempting to hypothesize that a similar pathway accounts for the maturation of a Cys-type sulfatase in *E. coli*, which possesses two AtsB-related genes.

We thus propose a new scheme for sulfatase maturation among prokaryotes (Fig. 6). In this new scheme, sulfatase maturation is based on oxygen-dependent (FGE) versus oxygen-independent (AtsB, CPE0635) machineries, rather than amino acid specific enzymes as currently stated (17, 27, 32).

We propose that AtsB and CPE0635 belong to the same family that we suggest to name anSME (*i.e.* anaerobic sulfatase maturation enzyme). In our proposed sulfatase maturation pathway, anSMEs are able to modify both types of sulfatases (*i.e.* Cys- and Ser-types), and FGE is strictly dedicated to the maturation of Cys-type sulfatases in aerobic organisms.

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