Membrane Topology of the Acyl-Lipid Desaturase from Bacillus subtilis*§

The Bacillus subtilis acyl-lipid desaturase (Δ5-Des) is an iron-dependent integral membrane protein, able to selectively introduce double bonds into long chain fatty acids. Structural information on membrane-bound desaturases is still limited, and the present topological information is restricted to hydropathy plots or sequence comparison with the evolutionary related alkane hydroxylase. The topology of Δ5-Des was determined experimentally in Escherichia coli using a set of nine different fusions of N-terminal fragments of Δ5-Des with the reporter alkaline phosphatase (Δ5-Des-PhoA). The alkaline phosphatase activities of cells expressing the Δ5-Des-PhoA fusions, combined with site-directed mutagenesis of His residues identified in most desaturases, suggest that a tripartite motif of His essential for catalysis is located on the cytoplasmic phase of the membrane. These data, together with surface Lys biotinylation experiments, support a model for Δ5-Des as a polytopic membrane protein with six transmembrane- and one membrane-associated domain, which likely represents a substrate-binding motif. This study provides the first experimental evidence for the topology of a plasma membrane fatty acid desaturase. On the basis of our results and the presently available hydrophobicity profile of many acyl-lipid desaturases, we propose that these enzymes contain a new transmembrane domain that might play a critical role in the desaturation of fatty acids esterified in glycerolipids.

The fatty acyl desaturases encompass a family of enzymes, representatives of which are found in all lower eukaryotes, plants and animals (for a review see Ref. 1), and some prokaryotes such as cyanobacteria (2) and bacilli (3), which have the function of introducing double bonds into fatty acyl chains. Although they all utilize molecular oxygen and reducing equivalents obtained from an electron transport chain, and the basic mechanism of the desaturation reaction may be very similar in all cases (4), fatty acid desaturases can be classified into three main subfamilies (1): (i) the soluble acyl carrier protein desaturases that introduce double bonds into fatty acids esterified to acyl carrier protein and are found in the stroma of plant plastids (4); (ii) the acyl-lipid desaturases, which are membrane-bound enzymes associated with the endoplasmic reticulum (1), the plant chloroplast membrane (5), the cytoplasmic membrane of some bacilli (6), and the plasmatic and thylacoid membranes of cyanobacteria (7) that desaturate fatty acids esterified in glycerolipids; (iii) the acyl-CoA desaturases, which introduce double bonds into fatty acids esterified to CoA (8) and are associated to the endoplasmic reticulum membrane of animals and fungi (1).

The soluble and the membrane-bound desaturases show different consensus motifs (for a review, see 4). Database searching for these motifs reveals that they belong to two distinct multifunctional classes of iron-dependent enzymes, each of which includes desaturases, hydroxylases, and epoxidases that act on fatty acids or other hydrophobic substrates (4, 9). The soluble class contains a consensus motif consisting of carboxylate and histidine ligands that coordinate an active site di-iron cluster, as revealed by the x-ray structure of the castor A3 stearyl-acyl carrier protein desaturase (10). The integral membrane class contains a different consensus motif for the putative active site composed of three histidine-rich regions, which are presumably involved in iron binding (9). Unfortunately, the structural information on membrane desaturases is scarce because of the technical limitations in obtaining large quantities of purified protein and the intrinsic difficulties in obtaining crystals from membrane proteins.

Membrane-bound desaturases belong to a superfamily of membrane iron proteins that share the purported iron ligand substrates (4, 9). Similar His-rich motifs are present in the bacterial membrane enzymes AlkB1 and xylene monoxygenase (9), which share strikingly similar residue spacings notwithstanding the different catalytic abilities of these enzymes. These residues are uniformly predicted to be located in the cytoplasmic face of the membrane, rather than in membrane-spanning domains (9). Because all the substrates for these enzymes are highly hydrophobic, they will likely partition into the lipid bilayer. In contrast, the electron donors for these enzymes are either soluble or peripheral membrane proteins.

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2 The abbreviations used are: AlkB, alkane hydroxylase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NHS-SS-biotin, sulfosuccinimidyl 2-biotinamidoethyl-1,3-dithiopropionate; PhoA, alkaline phosphatase; TMS, transmembrane segment; XP, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt.
(1). Overall, these features suggest that an active site assembled from these His-containing sequences may occur at or near the membrane surface.

The currently accepted structural model for membrane-bound desaturases was initially proposed by Stukey et al. (11) based in the sequences of rat and yeast \(\Delta^5\) stearyl-CoA desaturases. In this model, two long hydrophobic regions of 50 residues are predicted to span twice the membrane each (see Fig. 1). According to this model, most of the protein (240 of 334 residues for the rat \(\Delta^5\) desaturase) is located in the cytosolic side of the endoplasmic reticulum membrane, in two relatively long soluble stretches (11). This proposal was supported by the experimental determination of the topology of the membrane-bound AlkB from Pseudomonas aeruginosa (12). Later, Shanklin et al. (9) identified eight His residues essential for the function of the rat \(\Delta^5\) desaturase that are expected to bind the two iron ions in the active site. Five of these His residues are located in the cytosolic region flanked by these two transmembrane domains, whereas the remaining three His ligands are found in the soluble C terminal region (9) (see Fig. 1). Since this structural model was proposed by Shanklin et al. (9), many desaturase sequences were identified, but the model has not been challenged or supported directly by the experimental determination of the topology of any membrane-bound fatty acid desaturase.

We have reported recently the isolation and characterization of the des gene encoding the Bacillus subtilis membrane integral desaturase, \(\Delta^5\)-Des (6), which introduces a \(\Delta^5\) double bond at the \(\Delta^7\) position of the acyl chain of membrane glycerolipids (6, 13). This temperature-regulated acyl-lipid desaturase (14) exhibits relatively low sequence identity (typically about 23%) to membrane desaturases from cyanoacteria and plants (6). The B. subtilis desaturase contains all three histidine clusters found in the known membrane desaturases with the appropriate spacing (6). An additional, fourth His cluster (not found in other known desaturases) is located downstream from the third, conserved one (6). The amino acid sequence of \(\Delta^5\)-Des contains many hydrophobic stretches, consistent with the presence of several membrane-spanning domains. The hydrophathy profile of the deduced amino acid sequence of the des gene is similar to those of both \(\Delta^5\), \(\Delta^{12}\), and \(\Delta^9\) desaturases from cyanoacteria and to almost all known membrane-associated plant desaturases. Moreover, the spacings between each His-containing region and the end of the previous hydrophilic domain are conserved, as well, in the above mentioned acyl-lipid desaturases (6). Instead, the hydropathy plots of \(\Delta^5\)-Des, as well as of membrane-bound desaturases from different sources, show some differences with that of AlkB, which has provided the only structural model for \(\Delta^5\)-Des. To construct plasmids pET22-DES to study the desaturase topology of \(\Delta^5\)-Des through different experimental approaches that are finally aimed to understand the structure and function relationship in this family of enzymes.

In this paper we attempted to address the following issues:

(i) Are the His regions conserved in all desaturases equally essential in the B. subtilis \(\Delta^5\)-Des? (ii) Which is the topology of B. subtilis \(\Delta^5\)-Des? (iii) Are there different topologies in membrane desaturases and alkane hydroxylases? We present evidence that \(\Delta^5\)-Des is a polytopic membrane protein with six transmembrane- and one membrane-associated domain. In addition, we show that a tripartite motif of His essential for catalysis is located on the cytoplasmic phase of the membrane. Our results and the presently available hydrophobicity profile of membrane-associated desaturases from different sources now permit some discussion of a novel structure for acyl-lipid desaturases.

### Table 1

| Plasmid | Primer<sup>a</sup> | Sequence<sup>b</sup> |
|---------|------------------|------------------|
| pADH80  | H80f             | CAGATGCTCGGAGCfTCATCTTCCGATACAC |
|         | H80r             | GAAAGAACGTGACGAGCATCAGGAAGTAT |
| pADH116 | H116f            | TCGAATGCTGACGfTCATCTTCCGATACAC |
|         | H116r            | CTAGTTGCACTGATGGAATGCTGACG |
| pADH274 | H274f            | GCCAATGTTGGACGfTCATCTTCCGATACAC |
|         | H274r            | GAACGTCGGACGTAAATGACCCGATACAC |
| pADH275 | H275f            | GTACGCCAGTCTGTCCATTTAAGCCGATACAC |
|         | H275r            | CAAACTGCTGACGfTCATCTTCCGATACAC |
| pADH292 | H292f            | CTATAAGGCTCTGGACGfTCATCTTCCGATACAC |
|         | H292r            | GTGACGAGACTCAGGACGfTCATCTTCCGATACAC |
| pADH294 | H294f            | GAAAGGCTCTAGAAGCAGTCCAGGACGfTCATCTTCCGATACAC |
|         | H294r            | GCTGGAATCAGGGACGGATCCATTTAAGCCGATACAC |
| pADH295 | H295f            | GAAGTGCTCTAGAAGCAGTCCAGGACGfTCATCTTCCGATACAC |
|         | H295r            | gggaATGCTGACGfTCATCTTCCGATACAC |

<sup>a</sup> F and r indicate forward and reverse primers, respectively.

<sup>b</sup> Sequences are shown 5′ to 3′. Lowercase letters show variation from the wild-type sequence.

### EXPERIMENTAL PROCEDURES

#### Bacterial Strains and Growth Conditions

Escherichia coli strains DH5α and BL21 (DE3) were routinely grown in LB broth medium at 37 or 30 °C. Ampicillin and kanamycin were added at final concentrations of 100 and 30 μg/ml, respectively. The wild-type des gene, its site-directed mutations, or des-phoA fusions were expressed in E. coli BL21 (DE3) by induction with 1 mM IPTG. The B. subtilis strain used in this study was strain JH642 (trpC2, phe-I) and was grown in LB broth.

#### Site-directed Mutagenesis

Mutagenic oligonucleotides (Table I) were used to introduce nucleotide substitutions into cloned genes by overlap-extension PCR (15). In a first step we amplified overlapping fragments in separate PCR reactions. One fragment was obtained using the terminal primer DES-B (Table II) in conjunction with mutagenic reverse primers (Table I) and the other fragment using the terminal primer DES-C (Table II) in conjunction with mutagenic forward primers (Table I). The products were gel-purified and assembled in a PCR reaction primed with terminal primers only (DES-B and DES-C; see Table II) that introduce NdeI and XhoI sites, respectively. PCR fragments encoding modified des genes, digested with NdeI and XhoI, were cloned into the vector pET6a to generate plasmids pADn, in which n indicates the position of the histidine substituted by alanine in the \(\Delta^5\)-Des protein (Table I). These plasmids were sequenced to confirm the introduction of the mutation and to ensure that secondary mutations had not been introduced. In one of these plasmids (pAD-del), a stop codon was introduced at position 1990 of des, generating a C-terminal truncation of residues 294 to 352 of the primary sequence of Des.

#### Plasmid Construction

In all cases DNA fragments were obtained by PCR using the oligonucleotides described in Table II. Plasmids pET28-DES and pET22-DES were constructed as follows. The oligomers DES-B and DES-D (Table II), which generate NdeI and SalI sites, respectively, were used to amplify the wild-type des gene using as a template chromosomal DNA of strain JH842. The PCR product was cloned downstream of the T7 promoter on a pET28-a vector (Novagen) to yield plasmid pET28-DES. Plasmid pET22-DES was constructed by cloning the PCR fragment generated using primers DES-B and DES-D (Table II), which create NdeI and XhoI sites, respectively, in plasmid pET22-b (Novagen). This construction places a His tag at the C terminal of \(\Delta^5\)-Des. To construct plasmids pDBxxx, a sense primer specific for a 5′-coding region of des (DES-A; see Table II) and specific antisense primers to various regions of the same gene (DB52, DB73, DB109, DB126, DB150, DB186, DB214, DB230, and DB269; see Table II) were used to generate PCR products of appropriate N-terminal segments of des. Next, each PCR product was digested with NcoI and BamHI (restriction sites contained within the sense and antisense primers, respectively) and cloned downstream the T7 promoter of pET28-a (Novagen) to generate plasmids pDBxxx, in which xxx indicates the position of the amino acid residue in the protein Des coded by the GGA codon in the BamHI site.

The sequence coding for mature PhoA, lacking the 5′-segment coding for the signal sequence and the first five residues of the mature protein, was amplified by PCR using forward and reverse primers (PA1 and PA2; see Table II) that introduce BamHI and XhoI sites in the flanking regions, respectively. The BamHI-XhoI fragment was cloned in pDBxxx to create an in-frame fusion between the different length N-terminal sequences of PhoA and des.


**Table II**

| Oligonucleotide | Sequencea |
|-----------------|-----------|
| DES-A           | TACCTACCACTGgCTGAAACACATGTC |
| DES-B           | ATACCTGgATGACGAAMCAACCATCCTG |
| DES-C           | TTATCCgATGACGCTTCCGACGCTTCTG |
| DES-D           | ATATCCgATGACGCTTCCGACGCTTCTG |
| DB52            | GAGACTAGgAGCACTGGACGCTTGAAG |
| DB73            | GGGAAGgcTTAAAACTTTCGTCAGAAA |
| DB109           | GAACTGATGGgATGactccCTAATGAGAT |
| DB126           | GATGTCTGCGgATGATTTATTCGAGATT |
| DB150           | CTTAggATCTTTCAGAGCTTTGTCG |
| DB186           | GTAAGATGCgTTACAGCTTCTTGGC |
| DB214           | GAAATAAGgATGCGCTTACAGAATAG |
| DB230           | GATGTgATgGCAAAAGGCAACCAAC |
| DB569           | GAACGACgGATCCTAACAAGTgCCTGAGG |
| PA1             | GATCTCTACATAACgGCTTTGACG |
| PA2             | CACgGCCCgGCAgGTITATACCC |

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a Sequences are shown 5’ to 3’. Lowercase letters show variation from the wild-type sequence. Restriction sites are underlined.

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fragments of des and phoA (Δ5-Desxxx-PhoA fusions shown in Table III).

**Expression of Fusion Proteins—** *E. coli* BL21 (DE3) cells transformed with the pET28-a vector carrying the different Δ5-des-PhoA fusions (Table III) or the wild-type des gene were grown in LB medium supplemented with 30 μg ml⁻¹ kanamycin, until A₆₀₀ = 0.3. Then IPTG was added to a final concentration of 1 mM, and incubation was continued for another 3 h. Cells were collected by centrifugation, resuspended in loading sample buffer, and boiled for 5 min and then the samples were loaded onto SDS-polyacrylamide gels containing 10% polyacrylamide. After running the gels, the proteins were transferred to nitrocellulose for Western blotting. Δ5-Desxxx-PhoA fusion proteins were detected with monoclonal antibodies directed against PhoA, at a dilution of 1:5000. The Western blot was revealed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as detection substrates.

**The relative rate of synthesis of the fusion proteins was determined by densitometry of the relevant bands.**

**Alkaline Phosphatase Activity—** PhoA activity of *E. coli* BL21 (DE3) containing the Δ5-Desxxx-PhoA fusions or pET28-DES was detected in both plate or tube assays. Blue colonies on LB agar plates containing 40 mg ml⁻¹ X-1 and 1 mM IPTG indicate high PhoA activity, whereas white colonies indicate low PhoA activity. PhoA activity was also determined by measuring the rate of p-nitrophenyl phosphate hydrolysis essentially as described previously (16). Cells were grown overnight at 30 °C in LB broth, diluted 1:100 in 20 ml of fresh LB broth, and incubated at 30 °C with orbital shaking (200 rpm) until A₆₀₀ = 0.3. Then IPTG was added to a final concentration of 1 mM, and incubation was continued for another 3 h. At that point, 1-ml samples were extracted, and the A₆₀₀ and PhoA activity were determined. Specific activity was calculated according to the following formula: 1000 x (A₆₀₀/reaction time (min) x volume of cells (ml) x A₆₀₀) and was expressed in Miller units (17). The activities were corrected for the background activity measured with *E. coli* BL21 (DE3) harboring plasmid pET28-DES and were normalized by the rate of synthesis of the fusion proteins, determined by Western blot, as described above.

**Fatty Acid Analyses—** For measurement of fatty acid desaturation, *E. coli* BL21 (DE3) cells transformed with pET22-DES, containing the wild-type des gene, or plasmids containing the different des point mutants (pADEHn) were grown at 30 °C until A₆₀₀ = 0.3. Then 1-ml samples were labeled with 1 μCi of [1-¹⁴C]palmitic acid, and IPTG was added to a final concentration of 1 mM. Incubation was continued for 30 min. After incubation, the lipids were extracted from whole cells as described previously (13). The fatty acids of the glycerolipids were converted to their methyl esters with sodium methoxide and separated into unsaturated and saturated fraction by chromatography on 20% silica-gel impregnated silica gel thin-layer plates (13). Each lane contained ~120,000 cpm. The plates were developed at ~17 °C and autoradiographed.

**Topology Prediction—** Topology predictions were done using TOPPRED II (18), TMHMM (19), HMMTOP (20), and SOSUI (21). The results obtained from the different programs were qualitatively similar. Throughout this work, the specific results from TOPPRED II software will be shown and discussed.

**Cell Surface Biotinylation—** *E. coli* BL21 (DE3) cells transformed with plasmids pET22-b and pET22-DES were grown in LB medium until A₆₀₀ = 0.4 and then IPTG 0.5 mM was added for induction of des expression. After 2 h of induction at 30 °C (final optical density = 0.6), 4 ml of the culture were employed for the biotinylation tests. Cell surface proteins were labeled with the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Pierce), as described previously (22). Cells were incubated for 20 min at 4 °C with NHS-SS-biotin in alkaline medium, pH 8.5, the excess reagent was quenched with glycine, and the cells were dissolved in Triton X-100 and SDS. Biotinylated proteins were isolated from the cell extract with immobilized streptavidin, and the presence of His-tagged Δ5-Des was detected in the pool of surface proteins by 10% SDS-PAGE and Western blotting using an anti-His tag polyclonal antibody (Santa Cruz Biotechnology, Inc.). To establish that the reagent labeled only cell surface proteins, the biotinylation of the well characterized cytoplasmic protein GroEL was also determined using specific polyclonal antibody (23).

Streptavidin Blocking and Total Protein Biotinylation—Surface proteins were labeled as described. After washing the cells with glycine buffer to eliminate the excess of biotin, cells were incubated for 1 h at 4 °C with streptavidin, diluted 1:10,000 in phosphate-buffered saline. Cells were washed with phosphate-buffered saline and lysed with Triton X-100 and SDS, and biotinylated proteins were separated by immobilized streptavidin (Pierce).

Cells from another 4 ml- aliquot from the same culture were resuspended in 20 mM Hepes, pH 8.5, 2 mM CaCl₂, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and lysed with a French press. Total protein biotinylation was performed on this lysate for 20 min at 4 °C. After incubation, Triton X-100 was added until a final concentration of 1% to solubilize membrane proteins and eliminate cellular debris. Excess biotin was eliminated by ultrafiltration and buffer washing on an Amicon 8010 unit (Millipore), with a molecular mass cut off of 10 kDa. Afterward, the biotinylated proteins were isolated from this pool by using immobilized streptavidin and analyzed by SDS-PAGE 10% and Western blot.

**RESULTS**

**Topology Prediction of Δ5 Desaturase—** The hydropathy analysis of Δ5-Des, compared with that of AlkB (see Fig. 2A), was performed by using the program TOPPRED II, with the Kyte-Doolittle hydropathy scale and a window size of 21 residues. The Δ5-Des hydropathy plot (see Fig. 2A) predicts four clearly defined TMSs (residues 30–73 and 191–234), in agreement with the currently accepted model for the topology of the fatty acid desaturase family (9) (Fig. 1). Interestingly, the two first His-rich regions of Δ5-Des are located close to a region of relatively high hydrophobicity (residues 89–109). An additional hydrophobic stretch (153–173) can also be identified in the protein sequence. These regions are very good candidates for single-pass membrane-spanning helical segments (Fig. 2B, helices III and IV). According to this model, the second His-rich region would be located in the periplasmic space, thus precluding its involvement in the iron-active site. The presence of a fourth His cluster in Δ5-Des (not found in plant, mammalian, or cyanobacterial desaturases) located in the C terminus led us to speculate that a different alternative topology and active site formation could occur in the *B. subtilis* enzyme. We thus decided to determine the role of the different His-rich clusters.

**Site-directed Mutagenesis of Δ5 Desaturase—** Mutation of His residues that are proposed to be involved in iron coordination in the active site should in principle impair the ability of the enzyme to bind iron and abolish the desaturase activity (9). To investigate the role of the four His-containing motifs in the Δ5-Des, we made use of the ability of *E. coli* to functionally express the des gene (6). Because *E. coli* is not able to introduce double bonds into long chain saturated fatty acids, a test to evaluate whether any of the mutants of the des gene coded for active desaturases was performed by labeling *E. coli* cells, carrying the different plasmids, with radioactive palmitate and assaying the conversion of this fatty acid to cis-hexadecenoic acid (6).

We selected four His residues in the first three clusters (Fig. 2B, conserved residues His-80, His-116, and His-274 and the non-conserved His-275), which were mutated to Ala one by one
by using a PCR-based site-directed mutagenesis strategy (see “Experimental Procedures”). In addition, the three His residues located in the non-conserved cluster close to the C terminus (Fig. 2B, His-292, His-294, and His-295) were individually changed to Ala. The seven His-to-Ala point mutations into the des gene were expressed in E. coli, and the plasmid-encoded desaturase activities were assayed in vivo as described above.

All single mutations of residues His-80, His-116, and His-274 totally eliminated the in vivo activity, whereas mutations in any of the four non-conserved His residues (His-275, His-292, His-294, and His-295) still gave rise to an active enzyme, as indicated by the formation of cis-hexadecenoic acid from palmitic acid (Fig. 3). Synthesis of the wild-type and mutagenized desaturase forms of the proteins was verified by Western blot of whole cell extracts (data not shown). These findings suggest that the conserved residues His-80, His-116, and His-274 are essential for the activity of Δ5-Des. Therefore, the three His clusters conserved in all membrane desaturases are equally essential in this enzyme. Instead, the fourth His cluster does not seem to play a key role in building the iron-active site. These results strongly suggest that the His cluster flanking His-116 is involved in the enzyme active site and therefore cannot be located in the external face of the membrane but can be in the cytosolic side. We also explored the contribution of the C terminus to Des activity by assaying the ability of palmitate desaturation of a carboxyl terminal truncation (residues 294 to 352). The protein encoded by this mutant gene lacked desaturase activity (Fig. 3, lane 2).

Determination of the Topology of the Δ5 Desaturase by PhoA Fusions—The membrane topology of Δ5-Des was studied by constructing a series of fusion proteins with PhoA. The fusion joints were designed according to the generally accepted criterion (24), i.e. by locating most of them close to the C-terminal ends of the putative periplasmic and cytoplasmic loops according to the Δ5-Des model (Fig. 2B). In total, nine different fusion proteins were obtained (see details under “Experimental Procedures”), at positions 52, 73, 109, 126, 150, 186, 214, 230, and 269 (as indicated in Table III and Fig. 2B).

Fusion proteins were synthesized in E. coli BL21 (DE3) as verified by Western blot analysis using a monoclonal PhoA antibody. In all cases, the polypeptides were of the expected molecular weight (Fig. 4). PhoA fusion proteins with alkaline phosphatase activity indicate fusion to periplasmic loops, whereas fusion constructs with no alkaline phosphatase activity are characteristic of cytoplasmic domains. The chromosomal copy of alkaline phosphatase is not expressed when E. coli BL21 (DE3) is grown in rich medium, and cells appear as white colonies on plates containing the chromogenic substrate XP (25). PhoA activity of E. coli BL21 (ADE3) cells harboring the Δ5-Des-PhoA fusions was determined by blue/white screening on XP plates and by measuring the PhoA activity as indicated under “Experimental Procedures” (Table III).

Analysis of the activity of the Δ5-Des-PhoA fusion proteins and immunodetection of the expressed proteins results in the localization of the fusion proteins as summarized in Table III. Lack of alkaline phosphatase activity in fusion proteins Δ5-Des-PhoA 2, 6, 8, and 9 confirms the cytoplasmic location of the protein regions containing residues 73, 186, 230, and 269. The PhoA activity detected in the fusion constructs Δ5-Des-PhoA 1 and 7 supports the prediction that residues 52 and 214 are located in the periplasmic region. Instead, the result obtained with the activity of the fusion protein Δ5-Des-PhoA 3 positioned the second cluster of conserved His in a cytoplasmic segment, thus disproving the model resulting from our hydropathy analysis (Fig. 2B), as discussed below.

The lack of activity of the two fusion proteins Δ5-Des-PhoA 2 and 3 reveals that the sequence region between residues 74 and 109 is located in the cytoplasm and does not span the membrane as suggested by the predicted topology. On the other hand, the periplasmic location of residues 126 and 150 revealed by the fusion proteins 4 and 5 indicates that the stretch extending from residues 118 to 138 spans the membrane. We are thus able to propose the existence of a transmembrane domain not envisaged by the hydropathy plot, which we label as domain IIIa. The location of residue 186 (Δ5-Des-PhoA 6) in the cytoplasm confirms the existence of the transmembrane domain IV oriented toward the cytoplasmic side, which is coincident with the topology of the hydrophobic segment comprising residues 153–173 predicted by the hydropathy plot. Taken as a whole, our results indicate that the experimentally determined topology of Δ5-Des does not agree with either the current accepted model for desaturase topology or with the one pre-

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**TABLE III**

Properties of Δ5-Des-PhoA fusion proteins

| No. | Amino acid position | Fusion protein | MU* | XP plates | Fusion point location |
|-----|---------------------|----------------|-----|-----------|----------------------|
| 1   | 52                  | Δ5-Des52-PhoA  | 147 ± 30 | Blue | Periplasm |
| 2   | 73                  | Δ5-Des73-PhoA  | 2 ± 1.4   | White | Cytoplasm |
| 3   | 109                 | Δ5-Des109-PhoA | 16 ± 10  | White | Cytoplasm |
| 4   | 126                 | Δ5-Des126-PhoA | 173 ± 102| Blue  | Periplasm |
| 5   | 150                 | Δ5-Des150-PhoA | 140 ± 73 | Blue  | Periplasm |
| 6   | 186                 | Δ5-Des186-PhoA | 3 ± 2.4  | White | Cytoplasm |
| 7   | 214                 | Δ5-Des214-PhoA | 147 ± 2.2| Blue  | Periplasm |
| 8   | 230                 | Δ5-Des230-PhoA | 19 ± 2.9 | White | Cytoplasm |
| 9   | 269                 | Δ5-Des269-PhoA | 21 ± 9   | White | Cytoplasm |

* Miller units normalized as indicated under “Experimental Procedures.” These results are means of three independent experiments.

* Cells incubated in LB plates containing 40 mg ml⁻¹ XP and 1 mM IPTG.

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**Fig. 1. Topology model proposed for the rat stearoyl-CoA desaturase** (9). The cylinders indicate membrane-spanning segments. The location of the His residues conserved among Δ5 desaturase (NCBI accession number P07308) and alkane hydroxylase (accession number P12691) are indicated as open circles.

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predicted by hydropathy analysis of its amino acid sequence. The structural model that results from our experimental data is depicted in Fig. 5. This topology differs from the one expected from the model based in the analogy to alkane hydroxylase (Fig. 1) in that there are two additional TMSs (IIIa and IV). To validate the model proposed in Fig. 5, we decided to employ an
independent approach to probe the exposure of the fragment linking transmembrane regions IIIa and IV.

**Lysine Biotinylation**—Selective amino acid labeling with biotin represents an alternative approach to identify external loops. The possibility of applying this strategy depends on the location of key residues (generally Lys or Cys) that allows labeling of the loop between TMSs IIIa and IV and the model shown in Fig. 5 (we will not discuss these experiments on the model derived from hydropathy plots, shown in Fig. 2B, because it has been already discarded from our His mutagenesis analysis). According to the model shown in Fig. 1, Lys residues mentioned above should be located in the long cytoplasmic stretch between the hydrophobic regions. Instead, in the model proposed based in PhoA fusion experiments and His mutagenesis (Fig. 5), Lys-139 and -146 might be accessible to external reagents.

We tested the accessibility of lysine residues to reagents in the extracellular medium using the membrane-impermeant biotinylation reagent NHS-Ss-biotin. This reagent is particularly useful because of its high accessibility to small external loops, and it has been employed recently to characterize the external loop topology in the serotonin transporter (22). Cells were treated with this reagent and were then solubilized in detergent. Biotinylated proteins were extracted using immobilized streptavidin and analyzed by gel electrophoresis and Western blotting using an anti-His, polyclonal antibody. The same labeling and purification procedure was performed for lysed cells. The presence of biotinylated Δ5-Des was identified in the Western blots in both intact and lysed cells (Fig. 6). To discard eventual labeling of internal Lys residues in intact cells, we also controlled the biotinylation of cytoplasmic proteins by checking GroEL biotinylation, which was detected by blotting using specific anti-GroEL polyclonal antibodies. The presence of labeled GroEL protein in the Western blot analysis was detected mainly in lysed cells, suggesting that the positive results obtained for biotinylated Δ5-Des correspond to biotinylation of at least one Lys residue located in an external protein loop. This set of experiments confirms the outer location of the loop between TMSs IIIa and IV and the model proposed in Fig. 5.

**DISCUSSION**

This work provides crucial data for delineating the topology of a membrane-bound desaturase based on experimental grounds. First, we have identified the Δ5-Des essential His clusters involved in iron ligation by site-directed mutagenesis, which are coincident with those already identified in rat Δ9 and cyanobacterial Δ12 desaturases (9, 26). Our data also allow us to discard the possibility that the fourth His-rich region, which is found exclusively in the B. subtilis Δ5-Des, is involved in the active site. However, the C-terminal truncated mutant in which residues 294–352 were eliminated is inactive, indicating
that this region is essential either for desaturase activity or for the appropriate protein folding in the cytosol.

Second, through the analysis of protein fusion expression with alkaline phosphatase, we have been able to determine the membrane topology of the *B. subtilis* Δ5-Des. The results obtained with the Δ5-Des-PhoA fusions, as well as the systematic mutagenesis of conserved His residues, are fully consistent with the proposed model shown in Fig. 5. The polypeptide chain of Δ5-Des is oriented such that both the N and C termini face the cytosol, whereas the putative binuclear iron-active site (defined by the conserved His ligands) is located within several intervening membrane-associated domains.

Δ5-Des has six transmembrane domains: I (residues 30–50), II (residues 53–73), IIIa (residues 118–138), IV (residues 153–173), V (residues 191–211), and VI (residues 214–234). The model shown in Fig. 5 thus reveals the presence of two additional transmembrane domains when compared with the proposed topology of membrane desaturases, based on the sequence analysis of Δ5 stearyl-CoA desaturase and the experimental topology of AlkB (9, 12). Surface Lys biotinylation experiments on Δ5-Des confirmed that the loop located between the putative TMSs IIIa and IV is located in the periplasm, giving further support to the model derived from PhoA fusion experiments. On the other hand, the model shown in Fig. 5 does not fully agree with the hydrophathy plot from the deduced amino acid sequence of Δ5-Des discussed in the present work (Fig 2A). This theoretical analysis suggests the existence of TMS IV, but domain IIIa cannot be predicted accurately as a membrane-embedded region. Interestingly, the IIIa domain in Δ5-Des is clearly present in the hydrophathy plots of some Δ6 acyl-lipid desaturases, for instance, *Borago officinalis* (NCBI accession number AAD01410.1) and *Macor circinelloides* (NCBI accession number BAB69055.1) (see Supplemental Material).

In addition, our experiments show that the segment comprising residues 89–109 is located in a soluble portion of Δ5-Des rather than forming a TMS, as suggested by the hydrophobic plot shown in Fig 2A. Notably, this segment is located between the two first His clusters and might correspond to a protein region spatially located close to the iron-active site. A closer analysis of the hydrophathy plots of several acyl-CoA and acyl-lipid desaturases reveals the presence of such a hydrophobic stretch located between the two first His clusters. We therefore feel confident to propose that this region might be involved in substrate recognition. This segment is also present in AlkB (Fig 2A), which is able to hydroxylate medium-sized alkane molecules (12). Elucidation of the structural features able to shape the activity of similar di-iron-active sites toward double bond formation or hydroxylation of saturated alky chains is still a challenging issue that remains to be solved. Another relatively long hydrophobic stretch (residues 256–270) is positioned close in sequence to the third His-rich region, in the long cytoplasmic C-terminal portion of Δ5-Des (Fig 2A). The hydrophobic regions in the soluble portion should not be unexpected, because they may indicate protein regions that interact with the acyl chains of membrane lipids. These regions may represent a hydrophobic patch located close to the di-iron-active site, because it involves mostly residues located in the sequence next to the iron ligands.

The hydrophathy profile of Δ5-Des, as well as those of many glycerolipid desaturases from different sources, predicts five or six transmembrane helical segments (see Supplemental Material). However, alkane hydroxylases (12) and acyl-CoA Δ9 desaturases involved in the synthesis of monounsaturated fatty acids in yeast (11) and mammalian cells (27) lack the TMS 4 found in acyl-lipid desaturases. Apparently, the topographic difference found among membrane-associated desaturases could also be extended to mammalian desaturases involved in polyunsaturated fatty acid biosynthesis. The hydrophathy plots of murine and human Δ6 desaturase (accession number NP_062673, AAD 20018) predict only 4 TMS (28), whereas human Δ9 desaturase (NCBI accession number AAF 29378) shows a different profile to Δ5-Des (29). It has been reported that the substrate for rat liver microsomal Δ6 desaturase is linoleyl-CoA (30), whereas a Δ5 desaturase from a similar origin can directly desaturate 2-eicosatrienoic-phosphatidylcholine and 2-arachidonyl-phosphatidylcholine (31, 32). Thus, the presence of additional TMSs might be essential for desaturation of the acyl chains of membrane glycerolipids by stabilizing the structure and/or the activity of acyl-lipid desaturases. This study thus raises an interesting question about the functions of the cytoplasmic hydrophobic segments positioned close to the essential His in all membrane-associated desaturases and the TMS 4 domain found almost exclusively in acyl-lipid desaturases, which will require further experiments.

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