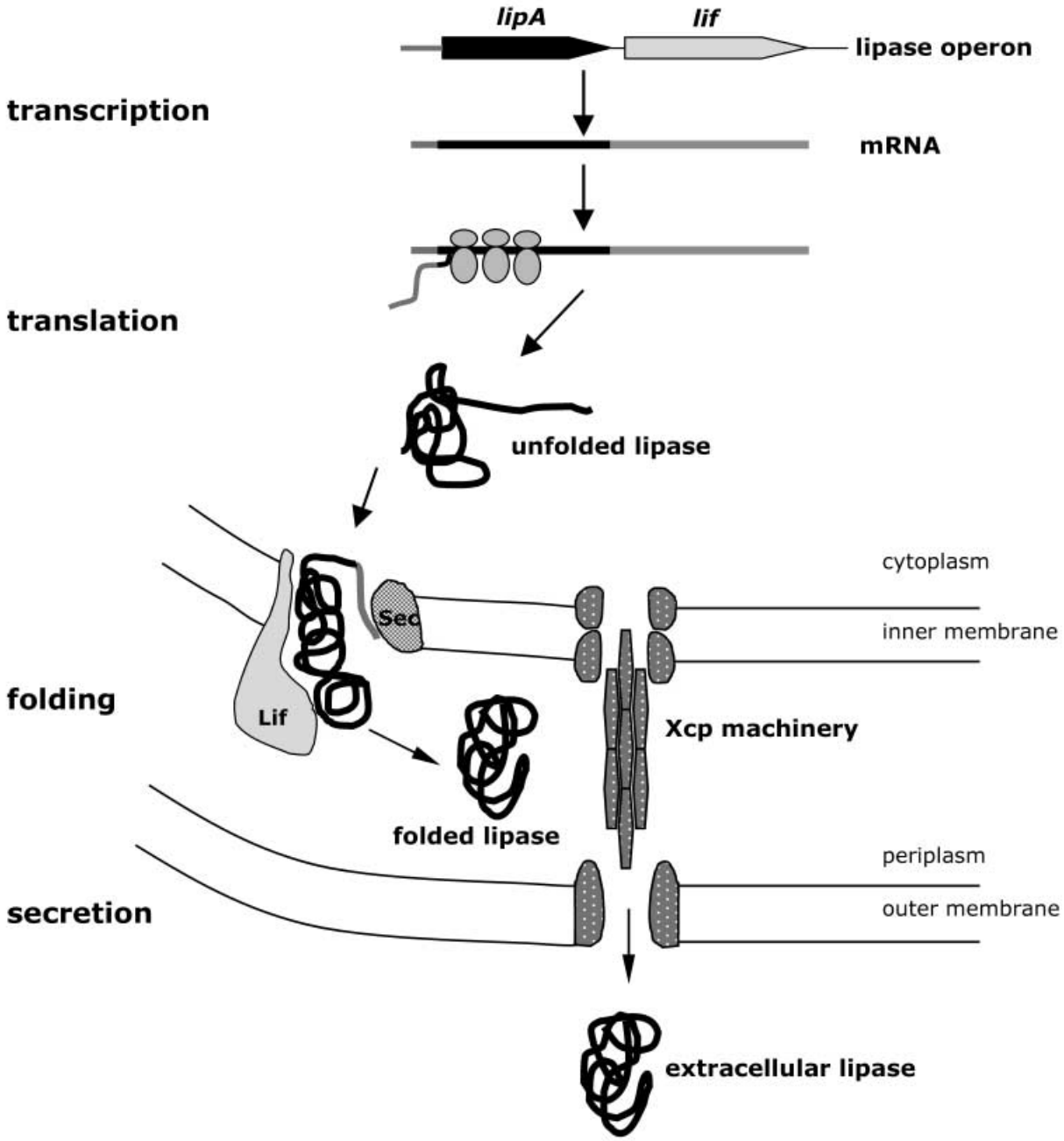
Lipase-specific foldases in bacteria



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Lipase-Specific Foldases

Frank Rosenau,[a] Jan Tommassen,[b] and Karl-Erich Jaeger*[a]

Lipases represent the most important class of enzymes used in biotechnology. Many bacteria produce and secrete lipases but the enzymes originating from Pseudomonas *and* Burkholderia *species seem to be particularly useful for a wide variety of different biocatalytic applications. These enzymes are usually encoded in an operon together with a second gene which codes for a lipase-specific foldase, Lif, which is necessary to obtain enzymatically active lipase. A detailed analysis based on amino acid homology has suggested the classification of Lif proteins into four different families and also revealed the presence of a conserved motif, $Rx_1x_2FDY(F/C)L(S/T)A$. Recent experimental evidence suggests that Lifs are so-called steric chaperones, which exert their physiological function by lowering energetic barriers during the folding of their cognate lipases, thereby providing essential steric information needed to fold lipases into their enzymatically active conformation.*

1. Introduction

In his pioneering work, Anfinsen demonstrated that the amino acid sequence of a polypeptide determines its three dimensional structure.[1, 2] However, Anfinsen also stated that protein folding might be influenced by other large molecules present in the same solution, which could include the same or other protein species or antibodies capable of intermolecular interactions.[2] The fact that a 100-amino-acid protein can adopt 10^{30} different conformations indicates that the theoretical number of possible conformations of a given protein is incredibly high. Although most of these conformations are wrong and unproductive, they can be stabilized by nonproductive interactions of the newly synthesized proteins with other cellular components. In living cells, such non-specific side reactions are prevented by two classes of folding modulators that have mainly been studied in bacteria:

1) Molecular chaperones, such as the DnaK-DnaJ-GrpE- or the GroEL-GroES- systems, which suppress off-pathway aggregations and facilitate proper folding by ATP-dependent cycles of binding and release of folding intermediates. The role of these intracellular molecular chaperones will not be described in this article; their structure and function has been the subject of several excellent review articles.[3–6]
2) Folding catalysts or foldases accelerate rate-limiting steps along the protein folding pathway.

Some proteins require the formation of the correct isomeric state of a certain peptide bond, for example, between a proline and its neighbouring amino acid. Such isomerization reactions are catalyzed by specific peptidyl-prolyl cis/trans isomerases (PPIases).[7] Furthermore, several proteins including extracellular enzymes often require the correct formation or isomerization of disulfide bonds, which is catalyzed in bacteria by periplasmic thiol-disulfide oxidoreductases named Dsb proteins.[8–11] Another unique class of chaperones exerts its function in the bacterial periplasm where they assist the correct folding of specific target proteins including bacterial pilus subunits,[12, 13] proteases[14] and lipases.[15] The PapD-like superfamily of chaperones has been extensively studied.[13] The X-ray structure of PapD itself, which is required for the assembly of P pili, was solved in a complex with its substrate, PapK.[16] The N-terminal propeptides of several proteases, including subtilisin, have been identified as intramolecular chaperones [IMCs], which mediate folding of their corresponding protease.[17] Upon binding to their cognate protease, the propeptides provide steric information required for proper folding of the enzymes. In addition, by binding to the enzymes' active site, they act as inhibitors, preventing premature protease activity before the enzyme is released in the extracellular medium. Similar observations were made with elastase from *Pseudomonas aeruginosa*, which possesses a propeptide that is essential for folding and secretion of the LasB protease. Like its α-lytic protease counterpart, the LasB propeptide is functional both in *cis* and in trans of the protease.[18, 19]

About a decade ago, it was found that the extracellular lipase gene *lipA* in the Gram-negative bacterium *Burkholderia cepacia* (at that time known as *Pseudomonas cepacia*) was encoded in an operon together with a second gene that was named *lipB* and shown to be necessary to lipase activity.[20] In the following years, several other LipB-like proteins were discovered in other Gram-negative bacteria and they all assisted the correct folding of their cognate lipases. Therefore, they were named Lifs to indicate that they constitute a unique class of *li*pase-specific *f*oldases.[15] Lifs are of outstanding interest because they are needed to fold the most important class of enzymes used in biotechnology and synthetic organic chemistry.[21] Besides the fungal lipases from

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Candida antarctica, *Rhizomucor miehei*, and *Candida rugosa*, the bacterial enzymes originating from *Pseudomonas* and *Burkholderia* species seem to be particularly useful for a wide variety of different biocatalytic applications including the enantioselective production of alcohols, amines, and carboxylic acids.[21–25] The production of enzymatically active lipases requires not only the presence of a cognate Lif but also the functional assistance of about 30 different cellular proteins before they can be recovered from the culture supernatant, indicating that folding and secretion of lipases, at least in these bacteria, are highly specific processes.[26]

Karl-Erich Jaeger was born in 1954, studied biology and chemistry at the Ruhr-Universität Bochum, Germany, and, under the supervision of Uli Winkler, obtained a Ph.D. in microbiology in 1982. From 1988 to 1989, he was a post-doc with Bob Hancock at the University of British Columbia in Vancouver, Canada. In 1995, he obtained his Habilitation from the Ruhr-Universität Bochum. In 2002, he became professor for molecular microbiology at the Heinrich-Heine-Universität Düsseldorf and director of the Institute for Molecular Enzyme Technology at the Research Centre in Jülich. His research interests are focused on bacterial enzymes and include the regulation of enzyme gene expression, mechanisms of folding and secretion, enzyme optimization by directed evolution and biotechnological applications.

Frank Rosenau was born in 1968. He obtained his diploma in biology in 1995 from the Ruhr-Universität Bochum, and a Ph.D. in microbiology in 2001 with a thesis supervised by K.-E. Jaeger. He currently directs the "Microbial Expression" research group at the Institute for Molecular Enzyme Technology at the Heinrich-Heine Universität Düsseldorf.

Jan Tommassen, born in 1952, studied biology at the Universiteit Utrecht. He did his Ph.D. thesis, entitled "Regulation and biogenesis of outer membrane PhoE protein of Escherichia coli *K-12", under the supervision of Ben Lugtenberg. After defending this thesis in 1982, he stayed at Utrecht, where he is now professor of Prokaryotic Microbiology. His main fields of research are the biogenesis of the Gram-negative bacterial outer membrane, protein secretion mechanisms in Gram-negative bacteria, and the structure/function relationships of outer-membrane proteins.*

2. Lif Proteins in Gram-Negative Bacteria

Lipases originating from *Burkholderia* and *Pseudomonas* species have been classified into subfamilies I.1 and I.2,[27] mainly based on differences in amino acid sequence homology. These lipases fold in the periplasm into an enzymatically active conformation and are subsequently transported through the bacterial outer membrane (see Figure 1) by means of a complex machinery consisting of up to 14 different proteins.[28] In *P. aeruginosa*, this so-called type II secretion machinery or "secreton" is built from 12 Xcp proteins and is required for the outer membrane translocation of several exoenzymes, among them exotoxin A, two phospholipases C, elastase LasB, and the lipases LipA and LipC.[29, 30] These enzymes first cross the cytoplasmic membrane by using either the Sec- or the Tat-pathway.[31] Then, they fold into an enzymatically active conformation in the periplasm, before they are translocated through the outer membrane.[32–34] However, to achieve a secretion-competent conformation, lipases require specific intermolecular folding catalysts, the Lif proteins.[15, 26] These foldases are usually encoded in the same operon together with their cognate lipases and have been identified in several *Pseudomonas* strains ,[35, 40] *B. cepacia*,[20] *B. glumae*,[41, 42] *Acinetobacter calcoaceticus*[43, 44] and *Vibrio cholerae*.[45] In addition, genes for Lif-related proteins are found in *Pseudomonas mendocina*, *Vibrio vulnificus* and the plant pathogens *Ralstonia metallidurans* and *Xylella fastidosa*. However, these proteins have only been identified in genome sequences by their homology to other Lif proteins, and there are currently no further experimental data available about their function. Interestingly, a lipase operon from *P. fragi* has been annotated which not only codes for a putative Lif but also for a novel lipase. This lipase differs significantly from the well-characterized *P. fragi* lipase, which does not require a Lif protein for folding.[46, 44]

Lifs represent a unique family of proteins without any significant homology to other classes of proteins. Some years ago, nine existing Lifs were classified into three distinct families.[44] Although Lifs from distantly related species share a significant degree of sequence similarity (see Figure 2 and Table 1], the level of sequence identity between members of this family is low, and we have identified only eight amino acid residues that are highly conserved in all known Lifs (Figure 2 a). In analogy to the previously published classification of *Pseudomonas* and *Burkholderia* lipases,[27] we propose here to classify Lifs into four distinct families I-IV (Figure 2 b and Table 1): family I comprises Lifs from *P. aeruginosa*, *P. mendocina*, *P. wisconsinensis* and *P. alcaligenes*; family II comprises Lifs from *B. cepacia*, *B. glumae*, *P. fragi*, *X. fastidosa* and *R. metallidurans*, and family III Lifs from *Acinetobacter calcoaceticus*. Lifs from *Pseudomonas* sp. strain KFCC10818,[40] *V. cholerae*[45] and *V. vulnificus* form family IV, because these proteins differ significantly from all other Lifs in size (279, 284 and 280aa) and amino acid sequence [Figure 2]. Prediction algorithms (PHD available on www.expasy.org[47])

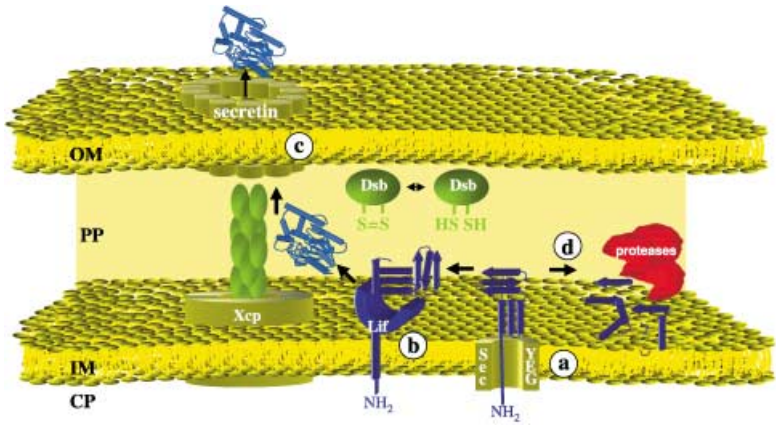


Figure 1. *Folding and secretion of* Pseudomonas aeruginosa *lipase. a) The lipase (indicated in blue) is synthesized as a precursor protein with an N-terminal signal sequence in the cytoplasm. It is directed to the inner membrane (IM) and translocated to the periplasm (PP) by the Sec-translocase complex of which SecY, E and G are the main components.[75] b) After the unfolded protein has passed the inner membrane, and the signal sequence is removed by signal peptidase, interaction with its Lif protein assists the lipase to adopt its enzymatically active conformation. This folding process is further assisted by accessory folding catalysts, for example, the Dsb proteins, which catalyse the formation of disulfide bonds.[9, 10] c) Finally, after folding is completed, the lipase is secreted across the outer membrane (OM) by the type II secretion machinery composed, in* P. aeruginosa, *of the Xcp proteins. These form a multisubunit translocation complex consisting of IM proteins and a number of so-called "pseudopilins", which supposedly form a periplasmic pilus-like structure that pushes lipase and other exoproteins through the outer-membrane pore formed by the secretin.[28, 33, 76] d) Alternatively, if correct folding fails, misfolded lipase is degraded by several periplasmic proteases.*

revealed that all Lifs seem to posses a very similar secondary structure, which is almost exclusively composed of α-helical (70%) and random coil (30%) elements. This structural conservation might be indicative not only of a common 3D structure, but also of the conservation of the catalytic mechanism.

The presence of a predicted hydrophobic transmembrane segment close to the N-terminus is a common feature of all Lifs (Figure 2a). Localization studies revealed that this segment anchors Lif to the bacterial inner membrane with almost the entire protein exposed to the periplasm.[42, 48] N-terminally truncated or modified Lifs still keep their ability to catalyze the folding of lipases indicating that the membrane anchor itself is not needed for the folding activity in vitro.[49–51] Moreover, we have constructed Lifs fused to cleavable signal sequences allowing their Sec-dependent translocation into the periplasm. Both *P. aeruginosa* and *B. glumae* Lif fusions were still able to activate their cognate lipases when they were expressed in the homologous host strain[52]. The uncleavable membrane anchor of Lifs may serve to prevent their secretion in complex with the lipases by the Xcp machinery.[52] Adjacent to the membrane anchor domain lies a proline- and alanine-rich stretch of amino acid residues with high sequence variability covering about 20% of the entire Lif protein. The family IV Lifs form an exception because they contain a large deletion in this region [Figure 2a]. Possibly, the main function of this segment is only to allow the C-terminal domain to protrude sufficiently from the membrane into the periplasm. In the large C-terminal domain of the Lifs, a more pronounced homology is observed. Several lines of evidence suggest that this domain is needed for interaction with the lipase and hence harbours the folding activity:

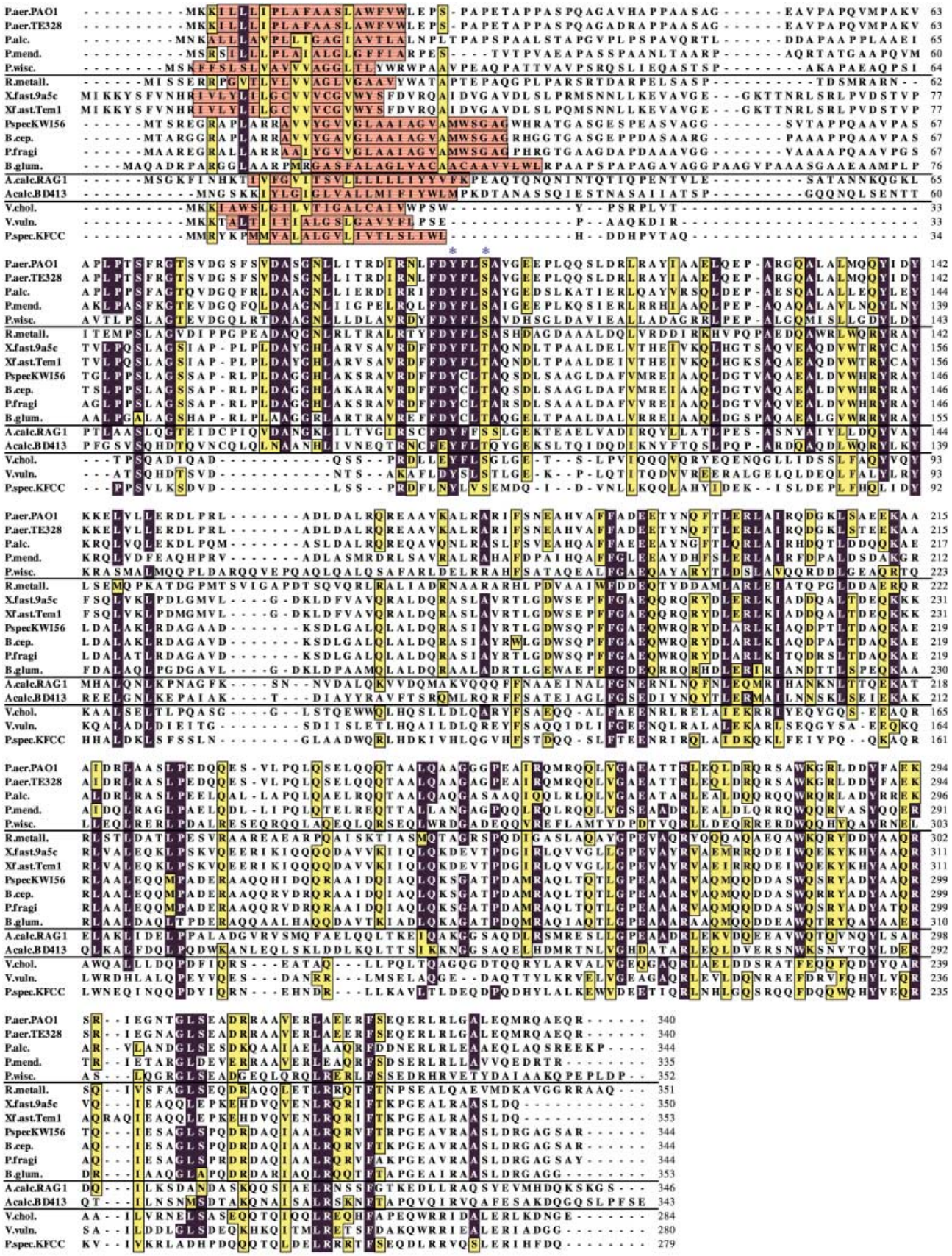
1) *P. aeruginosa* and *B. cepacia* Lif variants lacking almost the entire N-terminal domain (61 and 67 residues, respectively) were still functional in vitro.[49, 50]
2) After trypsin digestion of a *B. glumae* Lif–lipase complex formed in vitro, a 26 kDa Lif fragment was released which had lost the N-terminal 76 amino acid residues of the native Lif.[52] Since the Lif was completely degraded by trypsin in the absence of lipase, this result suggested that the large C-terminal domain had been protected in the complex by its interaction with the lipase.
3) Random mutagenesis experiments with the *P. aeruginosa lif* gene provided preliminary information about residues essential for Lif activity. Upon coexpression of a *lif* mutant library together with the lipase in *Escherichia coli*, inactive Lif variants were identified carrying mutations at positions Y99 and S102, both located in the C-terminal domain.[53] Interestingly, these positions are part of the conserved motif $Rx_1x_2FDY(F/C)L(S/T)A$ (Figure 2a), which we have identified to be present in Lifs of families I and II. Further mutagenesis studies are required to elucidate the role of this conserved motif in the folding process.

3. Physiology of the *P. aeruginosa* and *B. glumae* Lifs

3.1 Lipase–Lif interaction

The role of Lifs in the activation of lipases is difficult to investigate in vivo because folding and secretion seem to be tightly coupled cellular processes. The currently available knowledge mainly originates from genetic approaches, namely expression studies of different lipase/Lif systems in heterologous hosts. In addition, several experiments have been performed in vitro by using purified lipases and Lifs. These studies have clearly demonstrated that Lifs and their cognate lipases form stable complexes that can be copurified, coimmunoprecipitated or chemically cross-linked,[51, 54, 55] and that Lifs can mediate refolding of chemically denatured lipase in vitro.[37, 39, 48, 49, 56, 58]

A lipase gene and its cognate foldase gene usually form an operon suggesting a 1:1 ratio for both lipase and Lif expression. However, Northern blot analysis indicated that the primary transcripts of the *B. glumae* and *P. aeruginosa* lipase operons were subsequently processed leading to a major fragment corresponding to the lipase gene transcript only, whereas the *lif* part of the transcript seemed to be degraded.[42, 48] In fact, Lif proteins in *P. aeruginosa* and *B. glumae* were produced in significantly lower amounts than their cognate lipases. The same is true for the *P. alcaligenes* Lif, which led to the assumption that Lifs might act as multiturnover catalysts within the lipase-folding pathway.[52, 59] However, in vitro experiments revealed a 1:1



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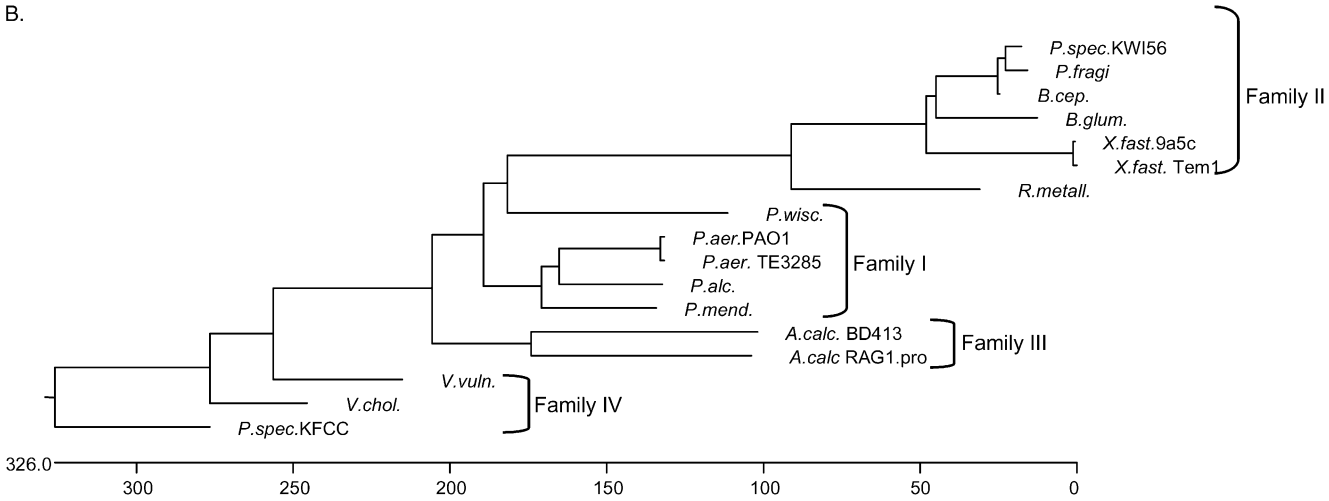

Figure 2. *Sequence comparison of the families I – IV of Lif proteins. Sequences were retrieved from protein and nucleotide databases by means of the Entrez server at NCBI (http://www.ncbi.nlm.nih.gov/Entrez/). Sequence -similarity searches were performed with the Blast 2.0 program.[77] Sequence comparison, sorting and alignment were obtained with the Match-box server[78] and the Clustal W program[79] by using the BLOSUM62 similarity matrix.[80] Further analysis and final presentation were done with the BioEdit program Version 5.0.9.[81] The phylogenetic tree was prepared by using the Megalign program from the Lasergene DNASTAR software package (DNASTAR, Madison, WI, USA).* ***A.*** *Sequence alignment of the Lif proteins. Families I – IV are grouped by solid black lines. Hydrophobic transmembrane segments were predicted by using the SAPS statistics software and are marked by light red boxes. Residues conserved in more than two families are marked with black boxes, and similar residues conserved in at least two families are shaded in yellow. Similarity shading was also based on the BLOSUM62 similarity matrix. The conserved residues Y99 and S102 identified as essential for* P. aeruginosa *Lif function are marked with blue asterisks.* ***B.*** *Phylogenetic tree. The tree is based on the 355-residue alignment shown in A. The length of the branches represents the distance between the sequences of Lifs from each pair of bacterial strains. The numbering at the bottom represents amino acid substitution events.* A. calcoaceticus *BD413 (A.cal.BD413),* A. calcoaceticus *RAG1 (A.cal.RAG1),* B. glumae PG1 *(B.glum.),* B. cepacia *(B.cep.),* P. aeruginosa *PAO1 (P.aer.PAO1),* P. aeruginosa TE3285 *(P.aer.TE3285),* P. fragi *(P.fragi),* P. mendocina *(P.mend.),* P. pseudoalcaligenes *(P.alc.),* Pseudomonas sp. *strain KFCC10818 (P.sp.KFCC),* Pseudomonas sp. *strain* KWI-56 *(P.spec.KWI56),* P. wisconsinensis *(P.wisc.),* R. metallidurans *(R.metall.),* V. cholerae El Tor *(V.chol.),* V. vulnifucus CMCP6 *(V.vuln.),* X. fastidosa Temicula 1 *(X.fast.Tem.1),* X. fastidosa 9a5c *(X.fast.9a5c).*

Table 1. *Classification of lipase-specific foldases from Gram-negative bacteria based on amino acid homology.*

Family no.	Origin of Lif	Identity [%]	Size Residues [kDa]	Amino acid composition [%] hydrophobic LIFVM	charged KRED	Acc. number
I	*P. aeruginosa* PAO1	100	340 (37.6)	25.0	26.2	CAA44998
	P. aeruginosa TE3285	100	340 (37.7)	24.7	26.2	Q01725
	P. pseudoalcaligenes	55	344 (37.9)	25.3	24.4	CAA02276
	P. mendocina	51	335 (37.3)	26.9	25.1	AAM14702
	P. wisconsinensis	30	352 (39.6)	24.1	25.6	O05938
II	*B. glumae* PG1	100	353 (36.8)	19.0	21.8	Q05490
	*P. spec.*KWI-56	57	344 (36.5)	19.5	21.8	P25276
	P. fragi	56	344 (36.3)	20.1	22.7	E04514
	B. cepacia	56	344 (36.4)	18.9	22.7	B39133
	X. fastidosa Temicula 1	46	353 (40.0)	29.7	26.1	NP 778696
	X. fastidosa 9a5c	46	350 (39.7)	30.6	26.0	NP 298472
	R. metallidurans	29	351 (38.6)	19.9	24.8	ZP 00025702
III	*A. calcoaceticus* BD413	100	343 (39.0)	25.4	21.6	Q43961
	A. calcoaceticus (RAG1)	32	346 (38.6)	28.3	21.4	Q9X2S4
IV	*V. cholerae* ElTor	100	284 (32.6)	24.3	21.8	NP 232621
	V. vulnificus CMCP6	41	280 (31.8)	27.5	28.2	NP 761197
	P. spec. KFCC10818	33	279 (33.3)	30.1	26.5	AAD22079

stoichiometry for lipase – Lif complexes during the activation process suggesting that Lifs function as single-turnover catalysts.[51, 55] Further evidence for this assumption was obtained by an in vivo approach in which the lipase – Lif system of *B. cepacia* was expressed in *E. coli*. The expression of the *lip* and *lif* genes was induced separately, and active lipase could only be obtained when Lif was synthesized first, that is, lipase expression was induced with a delay.[60] Moreover, when Lif expression was stopped, the lipase activation kinetics followed a sigmoidal curve, strongly indicating that Lif was used up during the folding reaction.[60] A possible explanation for these discrepancies is that Lif and lipase form a stable complex, which is only disrupted upon the secretion of lipase. Thus, in vitro or in *E. coli*, which does not possess an active type II secretion pathway, Lif remains associated to the lipase, allowing it to fold only a single lipase molecule. Nevertheless, the amount of Lif seems to be rate

limiting, since we have recently demonstrated that the cellular expression level of Lif is an important bottleneck for the production of lipase in *P. aeruginosa* and *B. glumae*. Overexpression in trans of the *lif* genes in both strains resulted in a considerable increase of extracellular lipase production.[58]

3.2 Lif specificity

Lifs have been shown to specifically activate only their cognate lipases. When wild-type Lifs from *B. glumae* and *P. aeruginosa* were coexpressed with the respective lipase from the other species, lipase folding did not occur.[55, 52] However, domain swapping of Lifs from *P. aeruginosa* and *B. glumae* resulted in hybrid Lifs, which could activate *B. glumae* lipase only when they contained a C-terminal segment of at least 138 amino acids of *B. glumae* Lif.[52] This result again suggests that the C-terminal domain is needed for the interaction of Lifs and lipases and may therefore determine the Lif specificity.

Lifs and lipases originating from closely related species can substitute for each other albeit with limited efficiency. When *P. aeruginosa* Lif was expressed in *P. aeruginosa* together with the lipase from *P. alcaligenes*, enzymatically active lipase was formed and secreted.[52] The same result was obtained when Lif-lipase combinations of *P. aeruginosa* and *P. wisconsinensis* were expressed from synthetic operons in *P. aeruginosa*, but *B. glumae* lipase could not be activated by *Pseudomonas* Lif, irrespective of expression in either *P. aeruginosa* or *B. glumae* [Rosenau et al., unpublished].

3.3 Regulation of *lif* gene expression

Lipases and their cognate Lif proteins are usually encoded in the same operon.[15] A remarkable exception was found in *P. aeruginosa*, which encodes an extracellular lipase LipC with 51% similarity to the lipase LipA. Whereas there is no Lif-encoding gene located in the vicinity of *lipC*, genetic studies revealed that the expression of enzymatically active LipC is strictly dependent on the presence of the Lif encoded in the *lipA* operon.[30] Expression of the *lipC* gene in *E. coli* resulted in the formation of inclusion bodies consisting of inactive LipC protein. When partly purified LipC from these inclusion bodies was subjected to in vitro refolding, enzymatic activity was observed only in the presence of purified Lif indicating that this Lif activates not only LipA, but also a second lipase, namely LipC (Figure 3). Interestingly, the expression of both lipase genes is differentially regulated in *P. aeruginosa*. A two-component system and a general regulatory protein regulate the expression of *lipA*.[26, 61] We have found that *lipC* is expressed also under conditions where *lipA* expression is totally repressed [Rosenau et al., unpublished]. However, since the formation of enzymatically active LipC requires the presence of Lif, this result suggested that the *lif* gene must be expressed independently from the upstream-located gene *lipA*. As a consequence, the existence of a novel promoter was predicted that should be located within the *lipA/lif* operon. Transcriptional reporter gene fusions with the *E. coli* β-galactosidase were constructed by using internal DNA fragments from the *lipA/lif* operon. These constructs allowed us to map the novel promoter to the 49-base pair intergenic region separating *lipA* and *lif* (Figure 4). Subsequent primer extension analysis performed with the *lif* mRNA has identified a transcriptional start site in the intergenic region [Rosenau et al., manuscript in preparation]. A more detailed analysis of the 49 base pair (bp) intergenic region between *P. aeruginosa* genes *lipA* and *lif*, revealed a remarkable content of repeated DNA sequences (Figure 4B). These direct and indirect repeats are predicted to form mRNA with characteristic secondary structural features like hairpin loops,[38] which may function as transcriptional terminators or processing sites for mRNA degradative enzymes during the expression of the lipase operon. Such intergenic hairpins have also been found within the *lip/lif* operons of *A. calcoaceticus*[43, 44] and *P. alcaligenes*[59] and have been suspected to be responsible for an increased production of lipase relative to Lif.

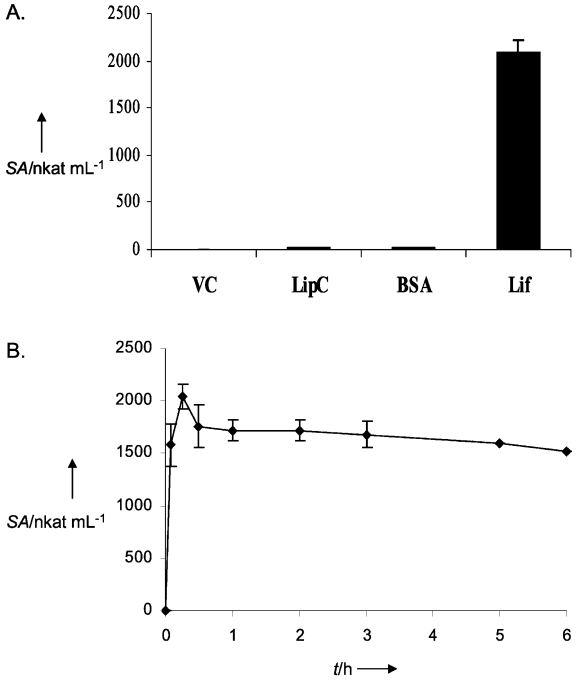

Figure 3. *In vitro activation of lipase LipC from* P. aeruginosa *by the Lif protein. The lipase gene,* lipC, *was expressed from the pET22blipC vector [Rosenau et al., submitted] and expressed in the commercially available T7* E. coli *expression strain, BL21(DE3) (Novagen, Madison). Lipase LipC inclusion bodies from crude cell extracts were chemically denatured with 8 M urea and subjected to refolding with purified Lif as described for lipase LipA.[10]* ***A.*** *Refolding of LipC depends on Lif. Crude cell extracts from* E. coli *BL21(DE3) harbouring the pET22b vector as control, (VC), show no activity after refolding in the presence of Lif. Extracts from cells expressing insoluble LipC denatured and refolded either in the absence of Lif (LipC) or in the presence of bovine serum albumine (BSA) as negative control, show only residual lipase activity. Significant lipase activity (specific activity, SA) was however obtained in the presence of Lif during refolding.* ***B.*** *Kinetics of the refolding experiments shown in Figure 3° A. Lipase activity was determined with p-nitrophenylpalmitate as the substrate. Error bars represent the standard deviation between three independent experiments.*

4. Molecular Mechanism of Lif Action

Chaperones that act by providing essential steric information to their target proteins, rather than by preventing off-pathway

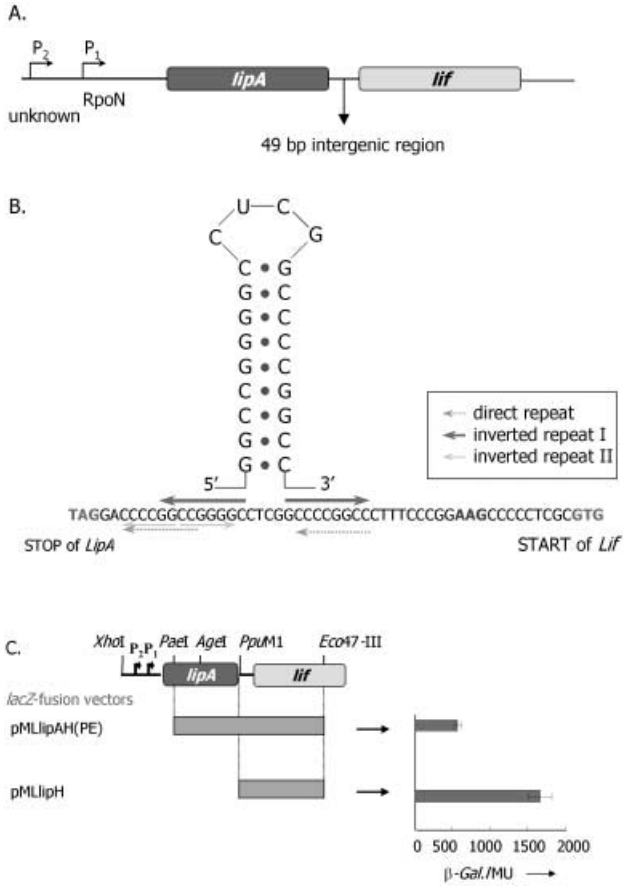

Figure 4. *Regulation of lif gene expression.* ***A.*** *The* P. aeruginosa *genes,* lipA *and* lif, *form an operon separated by a 49 bp intergenic region that is regulated at the transcription level by two independent promoters, P_1 and P_2. P_1-directed transcription depends on the alternative sigma factor σ^{54} (RpoN), and requires the presence of the two-component regulatory system LipQ/R,[61] the factors activating P_2 are not yet known.* ***B.*** *The 49 bp intergenic region located between the translational stop codons of the* lipA *gene and the downstream translation initiation codon of* lif, *contains direct and indirect repetitive sequences that potentially produce mRNA with stable secondary structures (hairpins). Such secondary structures may either influence the stability of the transcript or, alternatively, function as transcription terminators.* ***C.*** *Internal fragments of the lipase operon were used to create reporter gene fusions with the* E. coli *β-galactosidase gene, in the fusion plasmid, pML5.[82] The latter constructs were tested for promoter activity in* P. aeruginosa. *Although the known promoters, P_1 and P_2, preceeding the lipase operon were absent, the* lif *gene was still transcribed at a significant level demonstrating that Lif protein can be produced independently from its cognate lipase LipA. Error bars represent the standard deviation from three independent experiments. β-Galactosidase activity is given in Miller units.[83]*

reactions, have been designated "steric chaperones".[62, 63] Examples of steric chaperones include the propeptides of proteases, the *E. coli* PapD-like chaperones involved in pilus assembly, and the Lifs from Gram-negative bacteria.

The PapD superfamily of periplasmic chaperones is involved in assembling bacterial pili from individual pilus subunits (pilins), which fold in the periplasm and subsequently polymerize to form a fiber-like structure. Pilins have an unusual immunoglobulin (Ig)-like fold characterized by the absence of a C-terminal β-strand present in canonical Ig-like folds. The absence of this β-strand results in the exposure of a hydrophobic groove to solvent.[16, 64] In a complex between a pilin and a PapD-like chaperone, the Ig-like fold of the subunit is completed by a β-strand provided by the chaperone.[16, 64] This interaction has been named "donor strand complementation"; it stabilizes the pilins and suppresses their unproductive polymerization within the periplasm.[16, 64] Interestingly, the steric information missing in the pilin can also be provided by fusing the β strand of the chaperone to the C terminus of the pilin, which can then adopt a stable conformation even in the absence of the chaperone.[65] The stable complex of the chaperone and the pilin is resolved by interactions between the complex and the outer membrane translocation pore,[66] an event which initiates the subsequent polymerisation of the pilus fiber thereby preserving energy for the translocation of the assembled pilins across the membrane.[67, 68]

Many bacterial proteases, including subtilisin from *Bacillus subtilis* and α-lytic protease from *Aerobacter enzymogenes*, are initially synthesized with N-terminal propeptides, which function as intramolecular steric chaperones. In the case of α-lytic protease, the protease domain alone folds into a stable molten globule-like intermediate, which is, however, unable to convert to the enzymatically active native state in a biologically relevant time scale due to a large energetic barrier in the folding pathway.[69, 70] It should be noted that an energetic barrier in the folding pathway also implicates an energetic barrier for unfolding. Thus, even though energetically disfavoured over the thermodynamically more stable folding intermediate, the native state is kinetically trapped and prevented from unfolding by a substantial unfolding barrier.[70–72] The propeptide helps to overcome the energetic barrier in the folding pathway. It stabilizes the folding transition state and binds tightly to the native state, which in turn shifts the equilibrium to the propeptide/native state complex, since this complex is the thermodynamically most stable state.[71] Subsequently, the mature protease is released from this stable complex by degradation of the propeptide due its own catalytic activity.[72] The crystal structure of the propeptide/native state complex revealed the presence of an extensively hydrated interface between the propeptide and the protease domain.[73] It has been proposed that the relevant event in the stabilisation of the folding transition state and formation of the native state is the extrusion of bound water molecules, thereby achieving a higher degree of surface complementary between propeptide and protease. The extrusion of ordered water molecules would also increase the entropy of the system and explain the thermodynamic stability of the complex.[71]

In vitro refolding experiments have revealed that *B. glumae* lipase also folds in the absence of Lif into a stable native-like conformation, as shown by CD spectroscopy. This inactive conformation could be converted into the enzymatically active form by the addition of Lif, indicating that this chaperone helps lipase to overcome an energetic barrier in the productive folding pathway.[51] Further interesting parallels between the folding pathways of α-lytic protease and lipases include the apparent stability of the folding intermediates, which were found to be more stable than the native state indicating that the native states are not at their minimum free energy.[51] The propeptide of

α-lytic protease may contribute to expelling bound water molecules, thereby increasing the overall surface area of interaction and stabilizing the native conformation of the protease. For the *B. glumae* and *B. cepacia* lipases, successful in vitro refolding was described in the absence of Lif only when 40% glycerol was present in the refolding buffer.[51, 50] Glycerol is also thought to stimulate hydrophobic interactions within unfolded proteins.[74] However, refolding of *P. aeruginosa* lipase under the same experimental conditions proved unsuccessful unless Lif was added; this indicates that glycerol cannot be regarded as a general substitute for Lifs.

Interestingly, a single amino acid substitution was sufficient to create a self-folding variant of the lipase from *Pseudomonas* sp. strain KFCC10818. This variant was identified in a library of mutant lipase genes expressed in *E. coli* without the gene for the cognate Lif and was able to achieve an enzymatically active state.[40] The substitution probably lowers the energetic barrier in the folding pathway. Although it is not known whether any of the family I.1 and I.2 lipases[27] can be converted to Lif-independent folding variants by substitution of a single residue, one might speculate that such variants would have survived evolution if it were desirable for the cell to produce an enzymatically active lipase without the need for a specific chaperone. However, the fact that this does not seem to be the case stresses the importance of the Lif-dependent folding pathway. One explanation is that a decrease in the folding pathway energy barrier also implicates a lower energy barrier in the unfolding pathway and, thus, decreased stability of the lipase. Such a lower stability is disadvantageous, especially since most of the bacteria producing family I.1 and I.2 lipases also produce potent extracellular proteases, such as elastase in the case of *P. aeruginosa*. In this respect, it will be interesting to determine whether the Lif-independent lipase variant from *Pseudomonas* sp. strain KFCC10818 has increased protease sensitivity.

An alternative explanation for the evolutionary conservation of the Lif-dependent folding pathway is that Lifs are not only needed for the folding of their cognate lipase but also have additional functions within the living cell. It has been suggested that Lifs, in analogy to the protease propeptides, may function as lipase inhibitors after the folding is completed.[37] However, unlike proteases, which have to be tightly controlled until they have been secreted out of the cell, lipases exhibit no obvious harmful activities, since bacterial membranes are mainly composed of phospholipids, which are not substrates for lipases. Furthermore, the in vitro refolding experiments yielded active lipase that was still associated with the Lif; this demonstrated that the Lif did not inhibit lipase activity.[51] Another, more probable function of Lifs apart from their folding activity is a potential role in the secretion of lipases. In fact, a common secretion signal present in all type II-secreted exoenzymes is not yet known. It has been argued that this signal might be composed of structural elements of both the secreted enzyme and specific chaperones.[28] This would require an interaction of the lipase-Lif complex with components of the secretion machinery, which could in fact explain how the stable complex is resolved in vivo, because, unlike protease propeptides, the mature lipase cannot remove the Lif autoproteolytically.

5. Open Questions and Future Directions

Much knowledge has accumulated during the last few years regarding the gene regulation, physiological function, and potential mechanisms of Lif action. However, several important questions remain to be answered to completely understand the physiological role of Lifs and their potential application for the improvement of lipase production:

1) Do Lifs also function as enzyme inhibitors, thereby, preventing their cognate lipases to become active in the periplasm, and if so, what would be their natural cellular substrate?
2) What is the fate of Lifs after they have completed their foldase function?
3) Do Lifs have, in addition to their role in the folding of lipase, a direct role in secretion?
4) Do Lifs interact with other inner membrane proteins, for example, those forming the secretion machineries?
5) What does the Lif crystal structure look like?
6) Why do lipases need Lifs, whereas many other α-β-hydrolases fold without specific chaperones?

These questions indicate that research on Lif proteins will undoubtedly be intensified during the coming years, and the results will extend our knowledge on how living cells manage efficient folding and couple this to secretion of proteins.

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