The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase

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In mammals, lipoxygenases play key roles in inflammation by initiating the transformation of arachidonic acid into potent bioactive lipid mediators such as leukotrienes and lipoxins. In general, most bacteria are believed to lack lipoxygenases and their polyunsaturated fatty acid substrates. It is therefore of interest that an ORF (PA1169) with high homology to eukaryotic lipoxygenases was discovered by analysis of the whole-genome sequence of the opportunistic bacterial pathogen *Pseudomonas aeruginosa*. Using TLC and liquid chromatography-UV-tandem mass spectrometry (LC-UV-MS-MS), we demonstrate that PA1169 encodes a bacterial lipoxygenase (LoxA) that converts arachidonic acid into 15-hydroxyeicosatetraenoic acid (15-HETE). Although mammalian lipoxygenases are cytoplasmic enzymes, *P. aeruginosa* LoxA activity is secreted. Taken together, these results suggest a mechanism by which a pathogen-secreted lipoxygenase may modulate host defense and inflammation via alteration of the biosynthesis of local chemical mediators.

Pseudomonas aeruginosa is a medically significant Gram-negative bacterial pathogen noted for its high intrinsic resistance to antibiotics and for its ability to cause a wide spectrum of opportunistic infections (1). *P. aeruginosa* is perhaps best known for chronic lung infections that are the most significant cause of morbidity and mortality among cystic fibrosis patients (2). *P. aeruginosa* is also a cause of serious infections among immunocompromised cancer patients, burn patients, catheterized patients, and other hospitalized individuals (1).

Lipoxygenases (LO) are important regulators of host defense and inflammation in eukaryotes (3) but have not been characterized in prokaryotes. In general, LOs catalyze the stereospecific abstraction of hydrogen and insertion of molecular oxygen at specific fatty acid carbon–carbon double bond positions to form lipid hydroperoxides that are rapidly reduced to alcohols or further transformed to potent mediators (4–6) (Fig. 1*A*). For example, the leukocyte 5-LO initiates biosynthesis of leukotriene B_4, a potent chemoattractant for polymorphonuclear cells (PMN) and regulator of leukocyte trafficking. The 15-LO is a major LO in humans (4) and produces 15*S*-hydroperoxyeicosatetraenoic acid [15-H(p)ETE], which can be transformed by 5-LO into lipoxins, a class of bioactive eicosanoids that "stop" PMN infiltration and promote the resolution of inflammation (Fig. 1*A*) (5–7).

Because altered inflammatory states are a hallmark of certain *P. aeruginosa* infections (e.g., in cystic fibrosis) and because LOs are known to play key roles in the biosynthesis of inflammatory mediators, we were intrigued to find an ORF (PA1169) within the recently sequenced *P. aeruginosa* genome (8) annotated as a "probable lipoxygenase." Here, we report that PA1169 encodes a secretable arachidonate 15-lipoxygenase (LoxA), an enzyme activity generally believed to be absent from bacteria.

Materials and Methods

Bacterial Strains. *P. aeruginosa* strain PAO1 was obtained from V. Deretic (University of New Mexico, Albuquerque), PA14 from L. Rahme (Massachusetts General Hospital), PAK from S. Lory (Harvard Medical School), PA103 from J. Engel (University of California, San Francisco), and 39324 from the American Type Culture Collection (ATCC, Manassas, VA). PAK Δ*xcp*, which contains a complete deletion of the *xcp* gene cluster, was the kind gift of S. Lory and V. Tchesnokova (Harvard Medical School). Genomic DNA from various clinical and environmental isolates (MSH3, MSH10, MSHE2, CF18, CF5, 99-127, O2504, X24509, EDL, S54485, S35004) was the kind gift of S. Lory and B. Kulasekara (Harvard Medical School).

Recombinant DNA. The PA1169 ORF was amplified from PAO1 and PA14 genomic DNA and cloned into the *Kpn*I and *Xho*I sites of pBBR1-MCS4 (9). An in-frame deletion of *loxA* (Δ*loxA*) was introduced into the *P. aeruginosa* chromosome by standard allelic exchange. An N-terminal FLAG tag was inserted one amino acid after the presumed signal peptide cleavage site of LoxA by a two-step PCR and cloned into the *Kpn*I and *Xho*I sites of pBBR1-MCS4. A *loxA* promoter::*lacZ* transcriptional fusion was generated by cloning the *loxA* promoter region (−1098 to +349 where ATG = 1) from PAO1 and PA14 into the *Eco*RI and *Bam*HI sites of pBT0005 (B. Kulasekara and S. Lory, unpublished results). The fusion was transferred to the *loxA* locus on the PAO1 and PA14 chromosomes via a single crossover. The *loxA*::*lacZ* and Δ*loxA* alleles were confirmed by Southern blotting. All constructs were also confirmed by nucleotide sequencing.

Subcellular Fractionation and Western Blots. Stationary-phase cultures were diluted 1:100 in LB (plus carbenicillin if appropriate) and grown for 3 h with shaking at 37°C. Supernatant [dialyzed overnight at 4°C against periplasm buffer (50 mM Tris, pH 7.5/200 mM $MgCl_2$) or PBS] was concentrated 3-fold by using Amicon Ultra-15 filtration devices (Millipore) before LO assay. Periplasmic extracts were obtained as described (10, 11). Anti-FLAG mouse monoclonal antibody M2 (Sigma) or anti-β-lactamase rabbit polyclonal serum (Chemicon) were used to detect LoxA-FLAG or β-lactamase on Western blots of subcellular fractions.

Eicosanoid Analysis. Four microliters of ^{14}C-arachidonic acid [48 mCi/mmol (1 Ci = 37 GBq) Perkin–Elmer] (≈15 μM final) was added to a 500-μl sample containing enzyme, whole washed bacteria, and/or subcellular fractions (see above) and incubated for 45 min at 37°C with occasional vortexing. The soybean LO enzyme (250 units per reaction) was obtained from Sigma (L-3004). Enzyme reactions were extracted with 750 μl of acidified ether (30:4:1 ether:methanol:1 M citric acid) and spotted onto TLC plates (LK6D silica gel 60 Å, 250-μm thickness, Whatman). TLC plates were run at 4°C for 50 min by using

Abbreviations: LO, lipoxygenase; LC, liquid chromatography; MS-MS, tandem mass spectrometry; 15-HETE, 15-hydroxyeicosatetraenoic acid.

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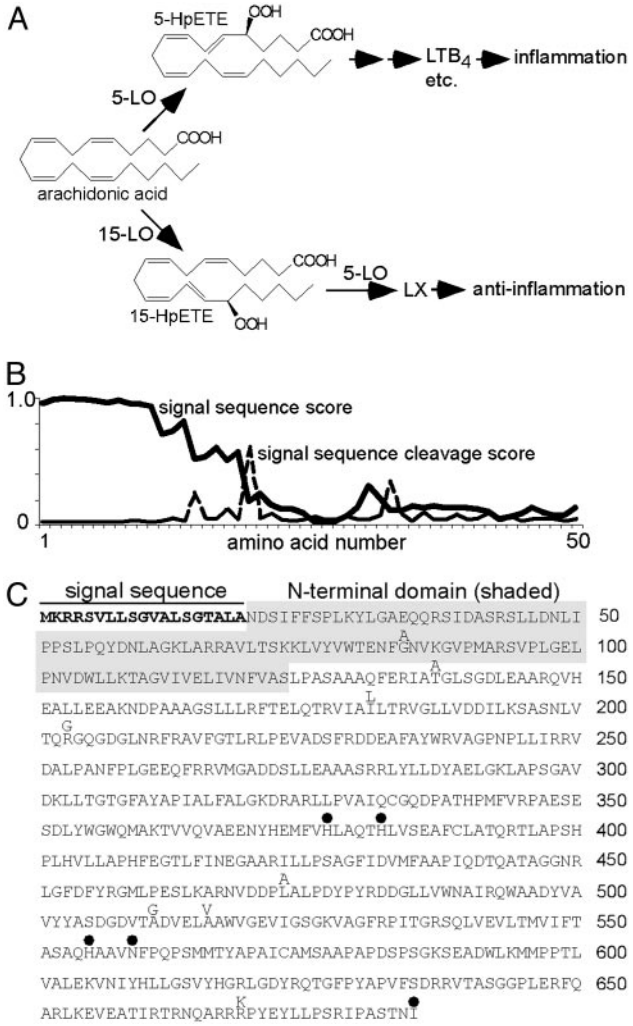

Fig. 1. (*A*) 5-LOs and 15-LOs dioxygenate specific carbons of arachidonic acid to form hydroperoxyeicosatetraenoic acids (HpETE), which can be rapidly reduced to hydroxyeicosatetraenoic acid (HETE, not shown) or can be transformed into other bioactive eicosanoids, e.g., leukotriene B_4 (LTB_4) or lipoxins (LX). (*B*) The first 50 aa of *loxA* were submitted to the SIGNALP server (www.cbs.dtu.dk/services/SignalP/) (13), and the signal peptide score and signal sequence cleavage score for each amino acid were plotted. As shown, authentic signal peptides should produce high scores for the signal sequence region until the peak for the signal sequence cleavage score, after which the signal sequence score should decline. (*C*) Inferred amino acid sequence of *P. aeruginosa* PAO1 ORF PA1169 (*loxA*). The predicted signal sequence is indicated in boldface. Based on alignments with the rabbit reticulocyte 15-LO, a putative N-terminal domain is shaded, as are conserved amino acids likely involved in coordinating a non-heme iron (filled circles). The eight amino acid substitutions in the PA14 LoxA sequence are indicated above the corresponding amino acid in the PAO1 LoxA sequence.

a mobile phase consisting of the organic phase of 110:50:20:100 ethyl acetate:2,2,4-trimethylpentane:acetic acid:water, as described (12). LO activity was calculated as the percentage of total radioactivity in the lane converted to the corresponding reduced alcohol product 15-hydroxyeicosatetraenoic acid (15-HETE).

Periplasmic fractions of *P. aeruginosa* PAO1 harboring pBBR-*loxA* or pBBR1-MCS4 (parental vector without *loxA*) were incubated with arachidonic acid (≈15 μM) (Cayman Chemical, Ann Arbor, MI) for 30 min at room temperature. The incubations were stopped by the addition of 2 vol of ice-cold methanol, followed by storage at −80°C. Products were extracted by using a C18 solid phase extraction cartridge (Alltech Associates) and subjected to liquid chromatography (LC)-UV-tandem mass spectrometry (MS-MS) for analysis as in ref. 7, by using a Finnigan LCQ liquid chromatography ion trap tandem mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with a LUNA C18-2 (100 × 2 mm × 5 μm) column and a photo-diode array detector that monitored UV absorbance. The analyte molecular anions and product ion mass spectra were collected for analysis. Physical properties including UV spectra, MS-MS, and coelution with authentic standards under matching experiments were used to establish structures.

Results

PA1169 (*loxA*) Is Homologous to Eukaryotic LOs. In the annotated release of the *P. aeruginosa* genome (8) (www.pseudomonas.com), the PA1169 ORF is described as a "probable lipoxygenase." Based on results presented below, we now assign the name "*loxA*" to PA1169. The most significant matches to LoxA in a TBLASTN search of the "nr" GenBank database were to eukaryotic LOs. The top two hits were to *Homo sapiens* and *Rattus norvegicus* arachidonate type 2 15-LOs. LoxA is 43.6% similar and 25.7% identical to the human 15S-Lox2 enzyme at the protein level. In general, mammalian LOs are classified according to which carbon of arachidonic acid they tend to oxidize. However, the positional specificity of LOs cannot be predicted based on protein sequence and must therefore be determined empirically (see below). Indeed, a tree dendrogram (Fig. 5, which is published as supporting information on the PNAS web site) suggests that LoxA is unique and is not clearly orthologous to a particular plant or animal LO. Typical of other LOs, the LoxA protein does not seem to contain transmembrane segments. However, the SIGNALP algorithm (13) predicts that the first 19 aa of the protein constitute a signal peptide followed by a signal-peptidase cleavage site (Fig. 1 *B* and *C*), suggesting that the protein may be secreted. This result is highly unusual for LO enzymes, which are generally cytoplasmic (4).

We constructed a transcriptional *lacZ* reporter fusion to the chromosomal *loxA* gene of PAO1 and PA14 to facilitate detection of *loxA* promoter activity. The *loxA* gene in PAO1 and PA14 seemed transcriptionally quiescent *in vitro* (LB) (Fig. 2*A*), a finding that was confirmed by quantitative Taqman RT-PCR and Northern blotting (data not shown). We used the *loxA*::*lacZ* reporter strain to test a variety of other *in vitro* growth conditions but were unable to detect significant *loxA* promoter activity under any *in vitro* growth conditions we tested. Nevertheless, the *loxA* gene was present in all of the following tested laboratory strains (PAO1, PAK, PA103, PA14), environmental isolates (MSH3, MSH10, MSHE2), cystic fibrosis isolates (CF18, CF5, 99-127), and other clinical isolates (O2504, X24509, UDL, S54485, S35004) as assessed by Southern blot and/or nucleotide sequencing. The entire ORF of *loxA* from the PA14 strain was sequenced, and the presumed amino acid sequences of *loxA* from PAO1 and PA14 are 98.8% identical (Fig. 1*C*). It is noteworthy that the *P. aeruginosa* LoxA seems to have retained the conserved amino acids that are involved in coordinating a non-heme catalytic iron atom present within mammalian LO (Fig. 1*C*). Because the *loxA* gene seems well-conserved, it seems likely to be expressed *in situ* in specific scenarios that seem not to be easily recapitulated *in vitro*.

***loxA* Encodes a Functional 15-LO.** To test whether *loxA* encodes a functional LO, we incubated [1-^{14}C]-labeled arachidonic acid with intact, washed *P. aeruginosa* (wild-type or Δ*loxA*) and analyzed the products using TLC. As expected from its weak *in vitro* expression, we found little if any specific ^{14}C-labeled LO products produced by wild-type PAO1, PA14, PAK, PA103, or ATCC 39324. Therefore, to prove that *loxA* encodes a protein with LO activity, the *loxA* ORF from PAO1 and PA14 was expressed from a low-copy plasmid vector. *Escherichia coli* and

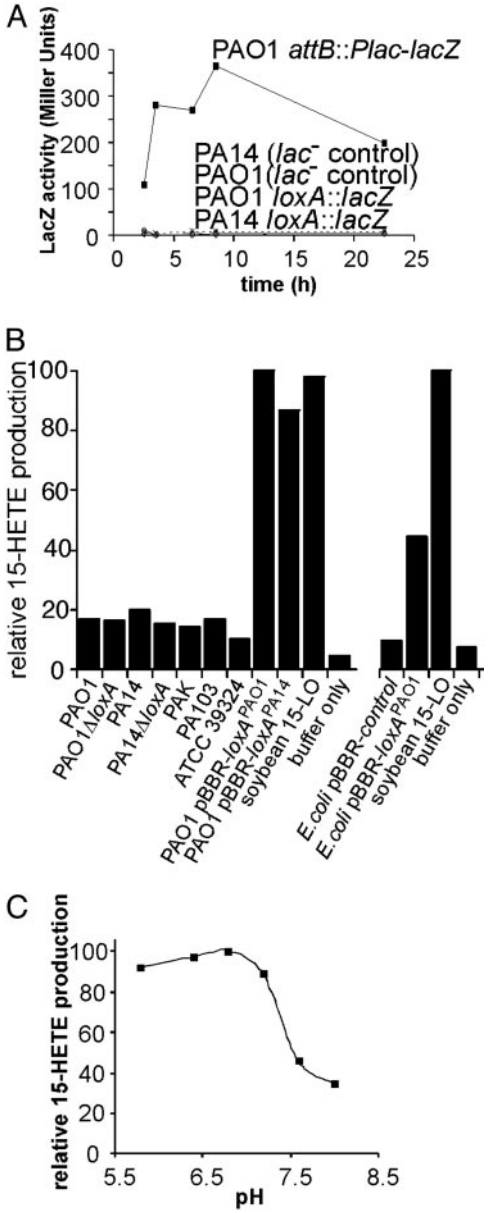

Fig. 2. (*A*) Low levels of *loxA* transcription *in vitro*. Overnight cultures of the indicated strains were diluted 1:100 into fresh LB and regrown with shaking at 37°C. At indicated time points, samples were taken for β-galactosidase assays. PAO1 *loxA*::*lacZ* and PA14 *loxA*::*lacZ* contain a transcriptional reporter fusion of *lacZ* (encodes β-galactosidase) to the chromosomal *loxA* gene of *P. aeruginosa* PAO1 and PA14, respectively. PAO1 and PA14 without the *lacZ* reporter were used as negative controls. PAO1 expressing a *lacZ* gene under the control of a constitutive *lac* promoter (integrated onto the chromosome at the neutral phage attachment site *attB*) was used as a positive control. (*B*) 15-HETE production by bacterial strains. Intact cells of the indicated bacterial strains were washed in PBS and incubated with [^{14}C]arachidonic acid. Lipids were extracted and separated by TLC to detect 15-HETE. Soybean 15-LO was incubated with [^{14}C]arachidonic acid as a positive control. A buffer-only sample (no bacteria, no enzyme) controlled for spontaneous oxidation products. pBBR-*loxA*^{PAO1} and pBBR-*loxA*^{PA14} are low-copy plasmids expressing the *loxA* gene of PAO1 and PA14, respectively, under the control of a *lac* promoter. The same plasmid or a control plasmid without insert (pBBR-control) was also transformed into *E. coli* strain SM10 as shown and tested in a separate experiment. (*C*) Apparent pH optimum for LoxA activity. Intact cells of *P. aeruginosa* PAO1 carrying the pBBR-*loxA*^{PAO1} plasmid were resuspended in potassium phosphate buffers of varying pH and incubated with [^{14}C]arachidonic acid. Lipids were extracted and analyzed by TLC. The percentage conversion of arachidonic acid to 15-HETE is plotted, with the percentage conversion measured in buffer of pH 6.8 taken as 100%.

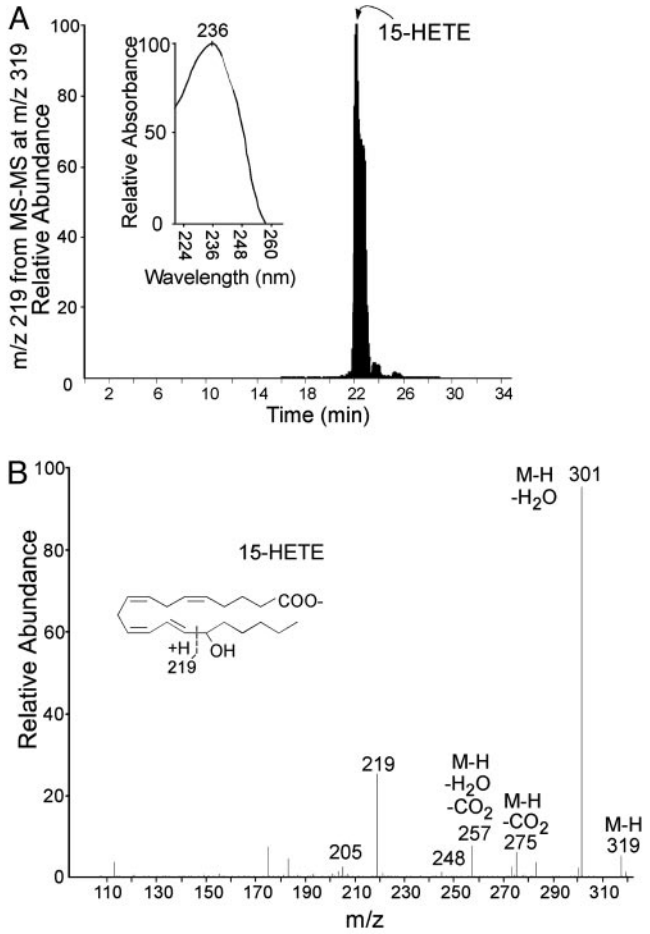

Fig. 3. LC-UV-MS-MS analysis of *P. aeruginosa* LoxA products. (*A*) The selective ion (at *m*/*z* 219 of MS-MS at *m*/*z* of 319) chromatogram showing 15-HETE and its in-line UV spectrum with absorbance maximum of 236 nm (*Inset*), characteristic of the conjugated diene in HETE. 15-HETE was identified based on its tandem MS-MS and UV spectra and LC retention time, as compared with authentic standards. (*B*) MS-MS spectra of the identified 15-HETE with diagnostic ions at *m*/*z* 219, 257 (M-H-H_2O-CO_2), 275 (M-H-CO_2), 301 (M-H-H_2O), and deprotonated parent ion 319 (M-H).

P. aeruginosa carrying the *loxA* expression plasmids produced specific ^{14}C-labeled products that comigrated with 15*S*-HETE standard (Fig. 2*B* and data not shown). Neither *E. coli* nor *P. aeruginosa* strains carrying an empty expression vector produced detectable 15-HETE (Fig. 2*B* and data not shown). Maximal conversion of arachidonate to 15-HETE by intact, washed bacteria was observed at approximately pH 7 (Fig. 2*C*). Bulk scale-up and purification of recombinant enzyme for detailed characterization were hindered by enzyme insolubility, possibly because the enzyme requires secretion for functionality (see below).

Tandem LC-UV-MS-MS Analysis of LoxA Products. The production of 15-HETE by *P. aeruginosa* LoxA was established by physical criteria by using LC-UV-MS-MS analysis (Fig. 3 *A* and *B*). The predominant HETE product detected was 15-HETE, which represented ≈95% of total monohydroxy products identified. Its MS-MS spectrum displayed diagnostic ions at *m*/*z* 219, 257 (M-H-H_2O-CO_2), 275 (M-H-CO_2), 301 (M-H-H_2O), and parent ion 319 (M-H) (Fig. 3*B*). Its UV spectrum exhibited an asymmetric single band of absorbance with a maximum at 236 nm (Fig. 3*A Inset*), characteristic of the presence of a conjugated diene structure. Lesser amounts of 12-HETE and other HETEs

were also identified (≈5%, data not shown). Material extracted from incubations with a control *loxA* deletion mutant (Δ*loxA*) revealed only trace amounts of mono-HETEs produced (data not shown). We conclude that *P. aeruginosa loxA* encodes an arachidonate 15-LO.

Secretion of LoxA Activity. Supernatant, periplasmic, and cell pellet (i.e., inner membrane and cytosolic) subcellular fractions from *P. aeruginosa* PAO1 expressing *loxA* from a low-copy plasmid were assessed for LO activity. Activity was found to be secreted to the periplasm and supernatant fractions. Marker enzymes [cytosolic β-galactosidase (Fig. 4*A*) and periplasmic β-lactamase (Fig. 4*B*)] were used to confirm the efficacy of our subcellular fractionation.

Next, we tagged LoxA with a FLAG epitope tag to follow LoxA protein localization. N-FLAG-tagged LoxA was as active as untagged protein and was secreted to the periplasm (Fig. 4*B*). However, nearly all N-FLAG-tagged protein was in the cytosol, as assessed by anti-FLAG Western blotting (Fig. 4*B*) and did not become active after disruption of the inner membrane by sonication. Hence, substrate accessibility is apparently not responsible for the inactivity of the cytosolic protein (Fig. 4*B*). Instead, the results imply that secretion of LoxA is likely coupled to its enzymatic activity.

To determine whether, indeed, LoxA was being actively secreted through the outer membrane and not merely "leaking" from the periplasm, cell fractions obtained from wild-type and Δ*xcp P. aeruginosa* were analyzed. The *xcp* genes encode a type II secretion system for exporting folded periplasmic proteins through the outer membrane (14). Supernatant from wild-type bacteria expressing *loxA* contained LO activity and produced 15-HETE whereas this activity was reduced by 84% in supernatant from Δ*xcp* bacteria (Fig. 4*C*). We conclude that, although LoxA activity predominantly localizes to the periplasm, a significant fraction of the active protein is also secreted extracellularly by an Xcp-dependent process.

Discussion

In this article, we identify and characterize a secreted arachidonate 15-LO from *P. aeruginosa*. Although polyunsaturated fatty acids, such as arachidonic acid, are widespread in mammalian cell membranes, *P. aeruginosa* (like most bacteria) produces little if any polyunsaturated fatty acid (15, 16). Consistent with an overall lack of polyunsaturated fatty acids, LOs have not been characterized in bacteria. A BLAST search of 134 completely sequenced microbial genomes (www.tigr.org) confirms that LOs are absent from most bacteria. Apart from *P. aeruginosa*, only two other microbes in the TIGR database (*Nitrosomonas europaea*, an obligate chemolithoautotroph, and *Anabaena* sp. strain PCC 7120, a cyanobacterium) seemed to contain LO-like sequences. In addition, two other microbes, whose genomes are not present in the TIGR database [*Sorangium cellulosum* (17), a soil bacterium, and *Nostoc punctiforme*, a cyanobacterium closely related to *Anabaena*] also seem to contain LO-like sequences. To date, none of these other putative bacterial LOs has been characterized. The absence of LO from most bacteria raises the possibility that LoxA may have been horizontally acquired from eukaryotes (17). The mechanism of such a putative horizontal transfer remains unclear. On the other hand, the acquisition of "eukaryotic"-like enzymes by *P. aeruginosa* is not unprecedented, in that a eukaryotic-like phospholipase D has also been described in *P. aeruginosa* (18).

Our present results account for several intriguing earlier findings. Sorrell *et al.* (19) reported that *P. aeruginosa* metabolizes arachidonic acid, but neither the products nor the enzyme responsible was identified. Other groups (20, 21), reported on the generation of mono-, di-, and trihydroxy derivatives of the fatty acids oleic acid and ricinoleic acid by *P. aeruginosa*; however, the enzyme responsible was not identified and arachidonic acid was not tested as a substrate.

LoxA seems to differ from mammalian LOs by virtue of an N-terminal signal sequence that targets LoxA for secretion. The

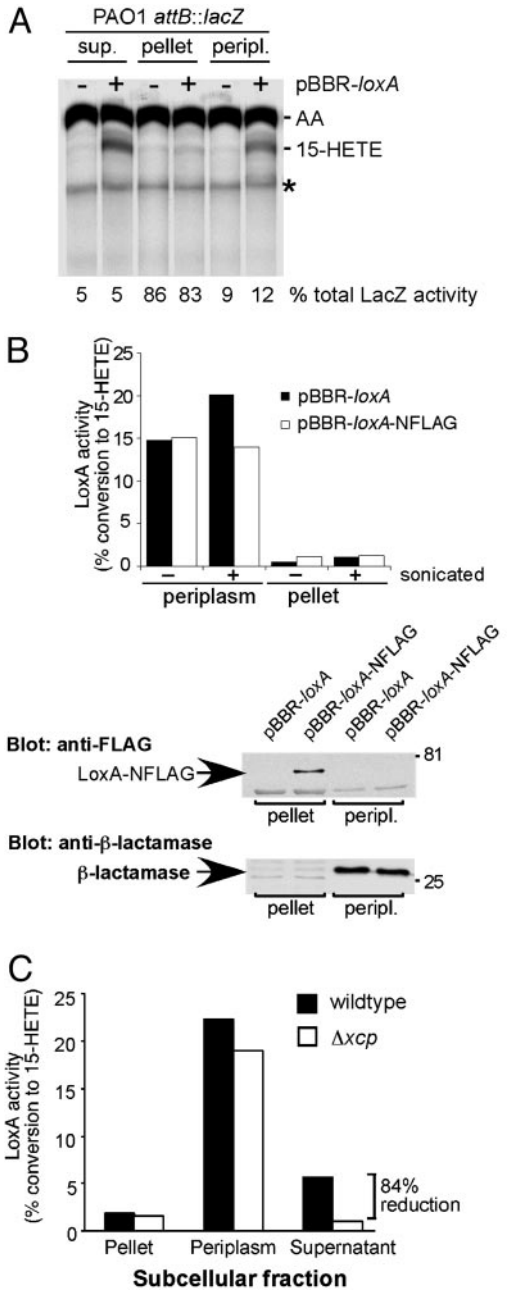

Fig. 4. Secretion of *P. aeruginosa* LoxA activity. (*A*) *P. aeruginosa* strain PAO1 *attB*::*lacZ* carrying a *loxA* expression plasmid (pBBR-*loxA*^{PAO1}) or control plasmid was tested for LO activity by TLC as in Fig. 2. The asterisk (*) indicates a nonspecific oxidation product routinely seen in all samples including buffer-only controls. A β-galactosidase assay was also performed on each fraction as a control for the fractionation procedure (β-galactosidase is a cytosolic enzyme). AA, arachidonic acid. (*B*) LoxA activity by LoxA-N-FLAG protein was assayed by TLC as in Fig. 2 and compared with the activity by untagged LoxA enzyme. Samples were sonicated where indicated. Samples from PAO1 pBBR-*loxA* and PAO1 pBBR-*loxA*-NFLAG were also subjected to Western blotting as indicated. (*C*) The pBBR-*loxA* expression plasmid was expressed in wild-type *P. aeruginosa* PAK or an isogenic Δ*xcp* mutant, and LoxA activity was assayed by TLC as in Fig. 2. LO activity is expressed as the percentage of ^{14}C-arachidonic acid converted to 15-HETE.

only other secreted LO described to date is the manganese-containing LO of the take-all fungus (a plant pathogen) (22), but this LO does not use arachidonate as a substrate. Nearly all LoxA activity seemed to be secreted, at least to the periplasm, with some activity further secreted to the extracellular milieu in a manner dependent on the Xcp type II secretion apparatus. It should be noted that even periplasmic enzyme is likely to be able to convert exogenous substrates for extracellular release because arachidonic acid and its mono-HETE derivatives (with molecular masses of 304.5–320.5 Da) should be able to cross the outer membrane of *P. aeruginosa*. Indeed, we observed that exogenous arachidonic acid could be converted to 15-HETE by intact washed bacteria (Fig. 2). Moreover, results from further experiments indicated that the majority of 15-HETE produced was cell-free and was not trapped within the bacterial periplasm (data not shown). Hence, *P. aeruginosa* 15-LO seems well-positioned to act on exogenous human-derived substrates, thereby potentially modulating the local inflammatory responses during *P. aeruginosa* infections.

Arachidonate is esterified in membrane phospholipids but can be deesterified by the action of phospholipase A_2. Estimates of the concentrations of free arachidonic acid in inflamed tissue range as high as 100 μM in the local milieu (23), levels greater than those used here in our *in vitro* studies. In addition, it has also recently been shown that the ExoU cytotoxin, secreted by certain *P. aeruginosa* strains, has phospholipase A_2 activity (24). The action of ExoU may therefore result in increased availability of arachidonic acid at local sites of *P. aeruginosa* infection.

15-HETE and its downstream products are known to have regulatory actions on immune and nonimmune cells. 15-HETE is readily transformed into lipoxins by infiltrating polymorphonuclear cells expressing 5-LO. Lipoxins down-modulate acute inflammation and are pro-resolution, even at nanomolar concentrations (25, 26). The role of eicosanoids and LOs in immune defense against pathogens is an area of continued interest (26, 27). Prior work has focused on the role of host-derived eicosanoids, that is, the chemical mediators themselves. Our present results demonstrate that at least one well-known pathogen, *P. aeruginosa*, is equipped to synthesize "eukaryotic" antiinflammatory chemical mediators. In this regard, it is significant that *P. aeruginosa* infections of cystic fibrosis patients are characterized by a nonresolving chronic lung inflammation. Interestingly, work presented in a complementary paper (28) demonstrates that *Toxoplasma gondii* also possesses a 15-LO, and that exogenous 15-LO is antiinflammatory *in vivo*. These observations raise the possibility that production of antiinflammatory lipid mediators may be a general strategy by which pathogens regulate the host–pathogen relationship.

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