The proteinaceous inhibitor of vertebrate lysozymes (Ivy) is produced by a collection of Gram-negative bacteria as a stress response to damage to their essential cell wall component peptidoglycan. A paralog of Ivy, Ivyp2 is produced exclusively by a number of pseudomonads, including Pseudomonas aeruginosa, but this protein does not inhibit the lysozymes, and its function was unknown. In this study, we demonstrate that the production of Ivy (homologs of both Ivyp1 and Ivyp2) correlates with bacteria that do not O-acetylate their peptidoglycan, a modification that controls the activity of the lytic transglycosylases. Furthermore, we show that both Ivy proteins are potent inhibitors of the lytic transglycosylases, enzymes involved in the biosynthesis and maintenance of peptidoglycan. These data suggest that the true physiological function of the Ivy proteins is to control the autolytic activity of lytic transglycosylases within the periplasm of Gram-negative bacteria that do not produce O-acetylated peptidoglycan and that the inhibition of exogenous lysozyme by Ivy is simply a fortuitous coincidence.

The vertebrate lysozyme inhibitor Ivy functions to inhibit the activity of lytic transglycosylase

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The PG sacculus is not a static structure, but rather it is continually expanded as cells grow. Its metabolism also involves its cleavage for the creation of both pores for secretion systems and sites for the insertion of flagella, in addition to the lysis of septa during cell division (3). A key class of endogenous enzymes responsible for PG lysis are the lytic transglycosylases (LTs) (4). The LTs cleave PG with the same specificity as the lysozyme, that is, the β-1,4-glycosidic bond between MurNAc and GlcNAc residues (Fig. 1). However, unlike lysozyme, LTs are not hydrolases as they cleave PG with the concomitant formation of an intramolecular 1,6-anhydromuramoyl reaction product (5).

The activity of both LTs and lysozyme is inhibited by modifications to the C-6 hydroxyl moiety of muramyl residues in PG. The most common modification to occur at this site among all bacteria is O-acetylation (Fig. 1). The O-acetylation of PG has been detected in a wide variety of bacteria, both Gram-positive and Gram-negative, including important human pathogens, such as Staphylococcus aureus, the enterococci (e.g. Enterococcus faecalis), Bacillus anthracis, Neisseria gonorrhoeae, and Neisseria meningitidis (6–8). In addition to this modification to the PG substrate, a collection of Gram-negative bacteria appears to produce small protein inhibitors of lysozyme, one of which is named Ivy (inhibitor of vertebrate lysozyme) (9).

First identified as the product of the ykfe gene in Escherichia coli (9), homologs and orthologs of Ivyc (Ivy from E. coli) have been found to be encoded on the chromosome of 21 other Gram-negative bacteria, each possessing the CKPHDC consensus subsequence (10). Of these, the homologous Ivyp1 from Pseudomonas aeruginosa also has been proven experimentally to inhibit type C lysozymes. Structural studies with Ivyc and Ivyp1 determined that the consensus sequence forms a rigid loop, which is locked by both a disulfide bridge between the two Cys residues and a salt bridge between the Lys and Asp residues. Ivy paralogs, which are found in species of Pseudomonas including P. aeruginosa, lack the consensus loop sequence, and it is replaced with a more variable sequence flanked by the two conserved Cys residues. Thus, despite their overall sequence similarity to Ivyc and Ivyp1, these Ivy paralogs do not inhibit lysozyme, and their function remained unknown. In this study, we demonstrate that both Ivyp1 and the paralog Ivyp2 inhibit membrane-bound lytic transglycosylase B (MltB) from P. aeruginosa and that the true physiological function of these periplasmic inhibitors may be to control autolysis in cells that do not O-acetylate their PG.
EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Unless stated otherwise, all reagents and chemicals were purchased from Sigma. The soluble derivative of recombinant MltB (sMltB) from P. aeruginosa PAO1 was isolated and purified as described previously (11).

Bacterial Strains and Growth—The sources of plasmids and bacterial strains used or constructed in this study are listed in supplemental Table 1. E. coli strains DH5α and BL21(DE3) were maintained on Luria-Bertani (LB) broth or agar (Fisher Scientific, Nepean, Ontario, Canada) at 37 °C, which was supplemented with 50 μg/ml kanamycin sulfate. For overexpression studies and the production of high levels of proteins, E. coli BL21(DE3) was grown in SuperBroth (5 g of sodium chloride, 20 g of yeast extract, and 32 g of Tryptone) at 37 °C with agitation.

Isolation and Purification of PG—Samples of insoluble PG were isolated from the different bacteria listed in supplemental Table 1 using the boiling SDS protocol, and they were purified by enzyme treatment (amylase, DNase, RNase, Pronase) as described by Clarke (12).

Determination of Extent of PG O-Acetylation—The extent of O-acetylation of soluble PG samples was determined as described previously (7) using the Megazyme acetic acid assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) to quantify the released ester-linked acetate (13). The extent of O-acetylation is presented as a molar percentage of muramic acid content.

Cloning of P. aeruginosa ivp1 and ivyp2—Genomic DNA from P. aeruginosa PAO1 was used as template for the PCR reactions. All oligonucleotide primers used in this study (listed in supplemental Table 2) were acquired from the Guelph Molecular Supercenter (University of Guelph, Guelph, Ontario, Canada). PCR amplifications were achieved in 50-μl volumes using a PerkinElmer Life Sciences GeneAmp PCR system 2400. Conditions were optimized for each primer pair using the Expand Long Template PCR system (Roche Applied Science, Laval, Quebec, Canada). Purification of PCR products was performed using the MinElute PCR purification kit (Qiagen, Mississauga, Ontario, Canada) or the High Pure PCR product purification kit (Roche Applied Science).

PCR products and vectors were digested with the appropriate restriction enzymes according to the manufacturer’s instructions and verified by agarose gel electrophoresis. The MinElute reaction cleanup kit was used to purify the DNA from these reactions prior to their ligation into the pET28a(+) vector. Individual constructs were isolated from transformants, screened for the correct size insert, and completely sequenced to confirm nucleotide identity.

Production and Purification of Ivyp1 and Ivyp2—E. coli BL21(DE3) freshly transformed with plasmid DNA was inoculated into SuperBroth supplemented with 50 μg/ml kanamycin and incubated at 37 °C until early exponential phase (A660 of ~0.5). Isopropyl β-d-thiogalactopyranoside (Roche Applied Science) was added to a final concentration of 1 mM, and growth was continued for a further 3 h at 37 °C. Cells were collected by centrifugation (5,000 × g, 15 min, 4 °C), washed, and resuspended in IMAC buffer (50 mM sodium phosphate buffer, pH 8.0, and 500 mM NaCl) containing Complete EDTA-free protease inhibitors, 10 μg/ml RNase, and 5 μg/ml DNase I. Suspensions were incubated on ice for 30 min prior to at least three passages through a French pressure cell press (18 000 p.s.i.). Insoluble debris were removed by centrifugation (5,000 × g, 15 min, 4 °C) and the supernatant was mixed with Ni2+-nitrilotriacetic acid-agarose (Qiagen) (0.5 ml/liter starting culture) for 1–5 h at 4 °C. Contaminating proteins were removed from the resin by washing with 10 column volumes of cold IMAC buffer, IMAC buffer at pH 7, and IMAC buffer at pH 6. Ivyp1 and Ivyp2 were recovered in 10 ml of IMAC buffer at pH 4.5 and dialyzed at 4 °C against 2 × 4 liters of 25 mM sodium phosphate buffer, pH 7.0.

Enzyme Assay and Inhibitions—The turbidometric assay of Hash (14) was used to monitor the time course of PG solubilization by either HEWL or sMltB. Whole cells or purified PG from Micrococcus luteus were suspended at a final concentration of 0.4 mg/ml in 25 mM sodium phosphate buffer, pH 5.8, containing 0.1% Triton X-100, 100 mM NaCl, and 10 μg/ml bovine serum albumin and subjected to brief sonication to provide homogeneous suspensions. Enzyme (20–60 μg) was added to 1.5-ml (final volume) samples of substrate suspension, and the decrease in turbidity was monitored continuously at OD660 nm, for a period of 15 min to 2 h. Reaction mixtures lacking the addition of enzyme served to control for settling of the insoluble substrate. The inhibition of HEWL and sMltB by...
Iivp1 and Iivp2 was tested by preincubating the enzymes with the potential inhibitors (0–15 μM) for 15 min at 25 °C prior to their addition to assay mixtures.

Other Analytical Techniques—Signal sequence predictions were performed using SignalP 3.0 and TMPred. Homology searches for homologs, orthologs, and paralogs of Ivyc were performed using the online BLASTP program to search the National Center for Biotechnology Information (NCBI) database, and analysis of sequence data was performed using ClustalW2 software (15). SDS-PAGE was performed using 12% acrylamide gels according to the method of Laemmli (16), and proteins were detected with Coomassie Brilliant Blue staining and Western immunoblot analysis using an anti-His6 tag antibody (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Protein concentration was determined by the BCA assay (Pierce) essentially according to the manufacturer’s instructions.

RESULTS

Identification and Phylogenetic Analysis of Ivy Homologs and Paralogs—A phylogenetic analysis conducted previously by Abergel et al. (10) found that Ivy appeared to be distributed sporadically and only among the Proteobacteria (Gram-negative bacteria). Despite the plethora of genome sequences that are now available, an exhaustive search of the publicly accessible databases using three sequences iteratively as the queries, those of E. coli Ivyc and P. aeruginosa Iivp1 and Iivp2, led to our discovery of only 13 more bacterial species as producers. Moreover, it appears that Ivy continues to be confined to a few species of the alpha (Glucanobacter, Paracoccus), beta (Burkholderiaceae, Neisseriaceae), and gamma (Enterobacteriaceae, Pseudomonadaceae) subdivisions of the Proteobacteria. Consistent with previous observations (10), each of the newly discovered hypothetical proteins are predicted to be localized to the periplasm.

A phylogenetic tree of Ivy homologs and paralogs is presented in Fig. 2 and, perhaps not unexpectedly, the proteins are organized generally according to phylogeny of the producing bacteria. Each member of the Ivyc/p1 family is characterized by the CKPHDC consensus sequence, which has been demonstrated to be essential for binding to C-type lysozymes (10, 17). The Iivp2 paralogs, represented by P. aeruginosa Iivp2 as the prototype, lack this consensus sequence but possess instead the consensus motif CEXXDC. The alignment of each of the hypothetical sequences is presented in supplemental Figs. 1 and 2. It is interesting to note that P. aeruginosa is still found to be the only producer of both Iivp1 and Iivp2 and that Iivp2 is confined to only the pseudomonads.

Analysis for O-Acetylated PG—Upon examination of the list of bacterial species that encode homologs of either Iivp1 or Iivp2 on their chromosome, it occurred to us that it included those few bacteria previously known to not O-acetylate their PG. Indeed, of the many bacteria that have been examined for the presence of O-acetylated PG (6–8), only E. coli and P. aeruginosa (7) have been found to be devoid of the modification. Thus, we conducted a search of each of the genomes of the Ivy-producing bacteria for the presence of either oatA (18) or the poa cluster (19, 20), genes known to encode the enzymes responsible for PG O-acetylation in Gram-positive and Gram-negative bacteria, respectively. Thus, S. aureus OatA (ZP_06334688), N. gonorrhoeae PatB (AAW89271), and B. anthracis Ape3 (NP843402) were used in these BLASTP searches. In addition, we also searched for homologs of N. gonorrhoeae Ape1 (AAW89270), an esterase responsible for removing O-acetyl groups from peptidoglycan (13) (Fig. 1) and also encoded within the poa cluster (19). Without exception, hypothetical homologs of these proteins were not found in any of the bacteria that would appear to encode homologs of either Iivp1 or Iivp2.

To confirm this absence, the PG from a selection of these bacteria was isolated and purified and then analyzed directly for O-acetylation. The list of these bacteria included strains of Klebsiella pneumoniae, Serratia marcescens, Yersinia enterocolitica subsp. enterocolitica, and Pseudomonas putida, in addition to P. aeruginosa and E. coli. With each, the level of ester-linked acetate associated with the isolated PGs was below 1% relative to muramic acid content; levels in producing species are greater than 15%, and typically range from 30 to 60% (6–8).

Production of P. aeruginosa Iivp1 and Iivp2—Appropriate oligonucleotide primers were synthesized for PCR amplification of both the 477-bp Iivp1 (PA3902) and the 483-bp Iivp2 (PA5481) from P. aeruginosa genomic DNA. Amplified products were cloned into pET28a(+), and the two constructs, pACCC-1 (encoding 165 amino acids; Iivp1 and a C-terminal His6 tag) and pACCC-3 (encoding 167 amino acids; Iivp2 and a
C-terminal His\textsubscript{6} tag), were transformed into \textit{E. coli} DH5\textalpha. To circumvent any potential problems associated with the production of multiple processed forms of the full-length proteins, truncated derivatives of Ivyp1 and Ivyp2, encoded on pACC-2 and pACC-4, respectively, were engineered that lack their N-terminal 24 and 23 amino acid residues, respectively. These residues were selected for deletion because they constitute their predicted cleavable signal sequences.

Recombinant Ivyp1 and Ivyp2 overproduced in \textit{E. coli} BL21(DE3) cells were isolated and purified by immobilized metal affinity chromatography using Ni\textsuperscript{2+}-nitrilotriacetic acid-agarose. The proteins were recovered by pH elution. Thus, lowering the pH of the wash buffer to 6 served to remove contaminating proteins, and both Ivyp1 and Ivyp2 were recovered in IMAC buffer at pH 4.5. Yields of ~3 mg of protein were routinely prepared from 1 liter of culture medium by this protocol.

**Ivy as an Inhibitor of Lytic Transglycosylases**—Ivyp1 from \textit{P. aeruginosa}, but not Ivyp2, has been shown previously to inhibit C-type lysozymes (10). This was confirmed in the current study. Preincubation of Ivyp1 at concentrations as low as 5 \textmu M resulted in complete inhibition of the lytic activity of hen egg white lysozyme (Fig. 3), whereas it remained fully active in the presence of Ivyp2. No other activity had been determined for Ivyp2, and consequently its function remained unknown.

Given that the LTs function as bacterial autolysins and share the same substrate specificity as lysozymes, we decided to test whether Ivyp2 acts to inhibit them. As shown in Fig. 3, purified Ivyp2 was found to inhibit the lytic activity of the soluble recombinant form of membrane-bound lytic transglycosylase B, sMltB, under conditions optimal for its activity (viz. 25 mM sodium phosphate buffer, pH 5.8, containing 0.1% Triton X-100 and 100 mM NaCl (11)). Moreover, and to our surprise, Ivyp1 also inhibited sMltB. These inhibitions were dependent on the concentration of each Ivy (Fig. 4). Careful titration of sMltB with increasing concentrations of either Ivyp1 or Ivyp2 revealed them to be equally potent inhibitors. Thus, 50% inhibition of sMltB was achieved with 3 molar eq of either Ivyp1 or Ivyp2, and less than 5% residual activity remained in the presence of an Ivy:sMltB molar ratio of 13.

**DISCUSSION**

The biochemical and structural analysis of the protein encoded by \textit{ykfE} from \textit{E. coli} was found to be a potent inhibitor of C-type lysozymes and thus renamed Ivyc for inhibitor of vertebrate lysozyme (10). Subsequent studies by others demonstrated that Ivyc and its homolog Ivyp1 from \textit{P. aeruginosa} contribute to both lysozyme resistance (22, 23) and survival of these bacteria in lysozyme-rich fluids, such as human saliva and breast milk, in addition to hen egg white (23). However, the essential cell wall component and target of lysozyme, PG, in Gram-negative bacteria is protected from exogenous agents by an outer membrane. Thus, the finding that (i) the production of Ivyc and its homologs is limited to Gram-negative bacteria rather than to Gram-positive bacteria, which possess exposed peptidoglycan and (ii) they are localized to the periplasm rather than to the external milieu was considered a paradox (10). Compounding this paradox is the fact that the Ivyc-like proteins have limited specificity, and they are weak inhibitors of only a subset of the G-type lysozymes (10, 17). Furthermore, despite its similarity, Ivyp2, which is produced by some pseudomonads, does not inhibit any type of lysozyme, and its function remained unknown (10). In the current study, we demonstrated that both the Ivyc homolog Ivyp1 and its paralog Ivyp2 function as inhibitors of the LTs, endogenous bacterial autolysins. Moreover, we observed that the sporadic production of Ivyc coincides with bacteria that do not O-acetylate peptidoglycan. In view of these findings, we suggest that the true physiological function of the Ivy proteins is to control the autolytic activity of LTs within the periplasm of Gram-negative bacteria and that the inhibition of exogenous lysozyme by the Ivy-like proteins is simply a fortuitous coincidence. This situation would thus be analogous to...
the initial identification and characterization of some housekeeping enzymes as antibiotic resistance factors. For example, the physiological function of the well studied gentamicin 2\'-N-acetyltransferase produced by *Providencia stuartii* was later identified as peptidoglycan O-acetyltransferase (24).

The LTs are ubiquitous bacterial enzymes (25) that cleave glycosidic bonds within the peptidoglycan sacculus to facilitate a number of critical cellular processes. Thus, they are required for, among other functions, the expansion of the cell wall by creating sites for the insertion of PG precursors, the turnover and recycling of PG, the cleavage of the septum during the separation of dividing cells, and the insertion of protein complexes that extend through the sacculus, such as secretion systems, flagella, and pili (recently reviewed in Ref. 4). As with the penicillin-binding proteins, peripheral membrane enzymes responsible for formation of new glycosidic linkages and peptide cross-linking in PG, bacteria produce a complement of different LTs (e.g. *E. coli* (26), and *P. aeruginosa* (25) produce seven and nine unique enzymes, respectively). They are classified as autolysins because if left uncontrolled, their activity results in the dissolution of the cell wall and consequent cell rupture. Given the essential requirement for a free C-6 hydroxyl group on muramoyl residues for the formation of the 1,6-anhydromuramoyl product (Fig. 1), the O-acetylation of PG has been proposed to provide a means of controlling LT activity in those cells that perform this modification (4). Although the O-acetylation of PG has been detected in many bacteria, both Gram-positive and Gram-negative, and include a number of important human pathogens, it is not universally found. Hence, it was recognized that other forms of control over the LTs would have to exist in those bacteria that lack O-acetylated PG, but nothing specific was known. In such cases, it was simply assumed that the physical association of the LTs as lipoproteins with the inner leaflet of the outer membrane and/or with the penicillin-binding proteins as synthetic complexes provided a level of control (4). However, this may not apply to the more soluble forms of LTs, such as Slt70 and Slt35 of *E. coli* or SltB1, SltB2, and SltB3 of *P. aeruginosa*. Thus, it seems reasonable to propose that because it is produced by Gram-negative bacteria that do not O-acetylate PG and it is localized specifically to the periplasm, Ivy serves to control the LTs of these bacteria.

Support for the physiological role of Ivy-like proteins in the control of LTs is provided by the observation that Ivy resides under the Rcs regulon in *E. coli* (27, 28). The Rcs phospho-relay system in *E. coli* was originally discovered as a regulator of capsule synthesis (29), but it was later found to be activated by the inhibition of PG biosynthesis following administration of \(\beta\)-lactam antibiotics (28). Mutational activation of the Rcs pathway leads to protection against \(\beta\)-lactam treatment (28), which, in fact, would result from the increased production of Ivy. Indeed, the lethal effect of Ivy production was shown to be dependent on the continued autolysis catalyzed by the LTs and other autolysins (21, 30, 31). Thus, the role of the Rcs phospho-relay in response to PG stress in some *Enterobacteriaceae* would be to induce the production of Ivy to prevent uncontrolled autolysis by LTs.

Currently, it is not clear why only *P. aeruginosa* produces both forms of Ivy1p and Ivy2p. It is possible that this requirement reflects the different complement of LTs that this bacterium produces when compared with the others. As noted earlier, *E. coli* produces seven distinct LTs (26), whereas *P. aeruginosa* appears to encode nine in its genome (25). However, a more significant difference between these two bacteria is that *P. aeruginosa* produces four different forms of MltB (11, 25), whereas *E. coli* only has one. Thus, this in combination with the larger complement of LTs in *P. aeruginosa* may necessitate the production of the two Ivy inhibitors.

Ivy inhibits HEWL by forming a tight complex involving a buried surface area of over 1,300 Å\(^2\) (10). Through this interaction, the active site cleft of HEWL becomes occluded by a loop protruding from Ivy, and a His residue at the center of this loop makes hydrogen bonds with both the catalytic acid/base Glu-35 and the catalytic nucleophile Asp-52. We have initiated crystallization trials with complexes of MltB and both Ivy1p and Ivy2p to determine whether a similar mode of inhibition occurs despite the fact that the LTs have only a single catalytic (acid/base) residue.

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