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The Plasmid-Encoded Regulator Activates Factors Conferring Lysozyme Resistance on Enteropathogenic *Escherichia coli* Strains[▽]

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We demonstrate that enhanced lysozyme resistance of enteropathogenic *Escherichia coli* requires the plasmid-encoded regulator, Per, and is mediated by factors outside the locus for enterocyte effacement. EspC, a Per-activated serine protease autotransporter protein, conferred enhanced resistance on nonpathogenic *E. coli*, and a second Per-regulated, *espC*-independent lysozyme resistance mechanism was identified.

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of diarrhea in young children, particularly in developing countries. The *l*ocus for *e*nterocyte *e*ffacement (LEE) pathogenicity island, present in all EPEC strains, encodes a type III secretion system (TTSS) and some TTSS effectors, as well as the intimin outer membrane protein and its translocated receptor (reviewed in reference 8). Studies have begun to elucidate the complex interactions of EPEC with host cells, particularly the LEE-mediated characteristic attaching and effacing pathophysiology (reviewed in reference 8). Typical EPEC strains, by definition, also have a large EPEC adherence factor (EAF) plasmid, which is absent in atypical EPEC. The EAF plasmid encodes type IV bundle-forming pili (Bfp), as well as a plasmid-encoded master regulator (Per) which directly or indirectly activates the transcription of LEE genes, *bfp*, and its own promoter (5, 25). There are other putative virulence genes on the EAF plasmid and at other locations outside the LEE, although many of these are present in only some EPEC lineages. Among them are *trcA*, a Rho GTPase present in EPEC2 lineage strain B171, and *espC*, a serine protease autotransported protein, which is encoded in a separate genomic island and is present in most EPEC1 strains, such as E2348/69 (23, 26).

Commensal, as well as pathogenic, *E. coli* have mechanisms for general and specific protection against antimicrobial peptides (1, 6), but the mechanisms by which EPEC avoid nonspecific host defenses remain largely unstudied. In this study, we evaluated EPEC resistance to C-type lysozyme, a 14.7-kDa antimicrobial peptide that is present in saliva at concentrations of about 40 μg/ml, as well as on skin, in breast milk, and in tears. Lysozyme is also secreted by goblet cells into the respiratory and intestinal tracts, where it can be detected in the mucus layer. The concentration of lysozyme in stool is in the range of 4 μg/ml (3). Lysozyme enzymatically cleaves β-1,4-glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan. Lysozyme is highly active against gram-positive organisms, and although it is less active against gram-negative bacteria, it demonstrates activity at physiological concentrations. We show that EPEC strain E2348/69 has multiple factors that confer exceptional lysozyme resistance and that these factors are outside the LEE and virulence plasmid but are under the control of Per.

The MICs of *E. coli* strains to recombinant human lysozyme (Sigma Aldrich) were determined by broth microdilution methods advocated for testing antimicrobial peptides (24). The MIC of lysozyme was two- to fourfold greater for EPEC strains E2348/69, C54-58, and B171 (20) than for *E. coli* K-12 strain MG1655 (Table 1). EPEC1 strains E2348/69 and C54-58 showed the greatest resistance and were consistently fourfold more resistant than *E. coli* K-12 (Table 1).

Observing that enhanced lysozyme resistance was not seen in the plasmid-cured variety of E2348/69 (Table 1), we hypothesized that Per, Bfp, or both might confer resistance. We therefore compared the survival of *per* mutant OG127 (11) following incubation with increasing concentrations of lysozyme to that of its isogenic wild-type strain, E2348/69. Antimicrobial killing was measured as the proportion of the inoculum surviving in the presence of lysozyme, human β-defensin-2, or human lactoferrin (Sigma-Aldrich) in peptide sensitivity assays, performed as described by Campos et al. (7). Although EPEC colonizes the intestinal mucosa, this organism is unlikely to be in niches richer in lysozyme, such as the saliva or the skin, for more than a short period; therefore, all assays were terminated at 1 h. Data were analyzed by an unpaired Student *t* test. As shown in Fig. 1A, 4 to 16 μg/ml of lysozyme decreased OG127 survival in lysozyme by more than 10% ($P < 0.05$) and this loss of resistance could be complemented by supplying the *perABC* genes on plasmid pINKper31 (20) in *trans*. These results suggest that Per substantially contributes to E2348/69 survival in lysozyme at physiological concentrations and temperature. Thus, as with peptide resistance in *Salmonella enterica* serovar Typhimurium and *Vibrio cholerae* (2, 12, 17), a virulence master regulator, in this case Per, activates factors that confer lysozyme resistance on EPEC.

Per activates the H-NS-like LEE-encoded regulator, Ler, which in turn induces the transcription of LEE operons encoding intimin, the TTSS, and LEE-encoded TTSS effectors, as well as factors outside the LEE (10). Per also activates the bundle-forming pilus (*bfp*) operon. We sought to determine which of the many *per*-regulated genes contribute to lysozyme resistance. Studies of other organisms have shown that factors

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TABLE 1. Lysozyme MICs of test *E. coli* strains

Pathotype	MIC (μg/ml)
E. coli K-12 MG1655	1.0
E. coli HB101	1.0
EPEC B171	2.0
EPEC C54-58	4.0
EPEC E2348/69	4.0
Plasmid-cured variety of E2348/69 (JPN15)	1.0

that confer autoaggregation or are required for biofilm formation can contribute to antimicrobial-peptide resistance (21). We hypothesized that *bfpA*, an EAF gene encoding the subunits of the bundle-forming pilus which mediates the attachment of EPEC to the enterocytes, as well as autoagreggation and biofilm formation (19), might contribute to lysozyme resistance, particularly as Bfp proteins are expressed early in EPEC pathogenesis. Since an EPEC Ler mutant demonstrates pleiotropic effects, including the expression of other pili (10), we opted to examine factors downstream of Ler rather than to evaluate a Ler mutant. In particular, we reasoned that an *escN* mutant with a disabled TTSS might show lysozyme sensitivity if any secreted effector proteins contribute to lysozyme resistance. As shown in Fig. 1B, mutants UMD901 (E2348/69 Δ*bfpA*) (28) and CVD452 (E2348/69 Δ*escN*) (14) showed lysozyme resistance comparable to that of wild-type E2348/69 at all test concentrations ($P > 0.05$). Thus, we concluded that neither Bfp nor any TTSS virulence factors contribute to E2348/69 resistance to lysozyme.

Ler, the LEE-encoded regulator that is activated by Per, also activates *espC*, a gene encoding a serine protease autotransporter protein, which is located on a separate pathogenicity island in a subset of EPEC strains (10, 18). EspC has previously been shown to have enterotoxin activity in rat jejunal tissue mounted in Ussing chambers and, unlike other EPEC-secreted proteins, EspC secretion is TTSS independent (10, 14, 18, 23). EPEC strains E2348/69 and C54-58, in which we recorded the highest lysozyme MICs (Fig. 1A), are *espC* positive, while strain B171 is *espC* negative (18). We used pJLM174, an *espC* clone under the control of the arabinose promoter, to study lysozyme resistance in a neutral *E. coli* K-12 background (18). In the presence of 0.2% arabinose, sufficient EspC is expressed and secreted into culture supernatants to be visualized by Western blotting and to produce serine protease and enterotoxin activity (9, 18). No EspC or EspC-related activity is detected in the presence of 2% glucose. Upon induction, we were

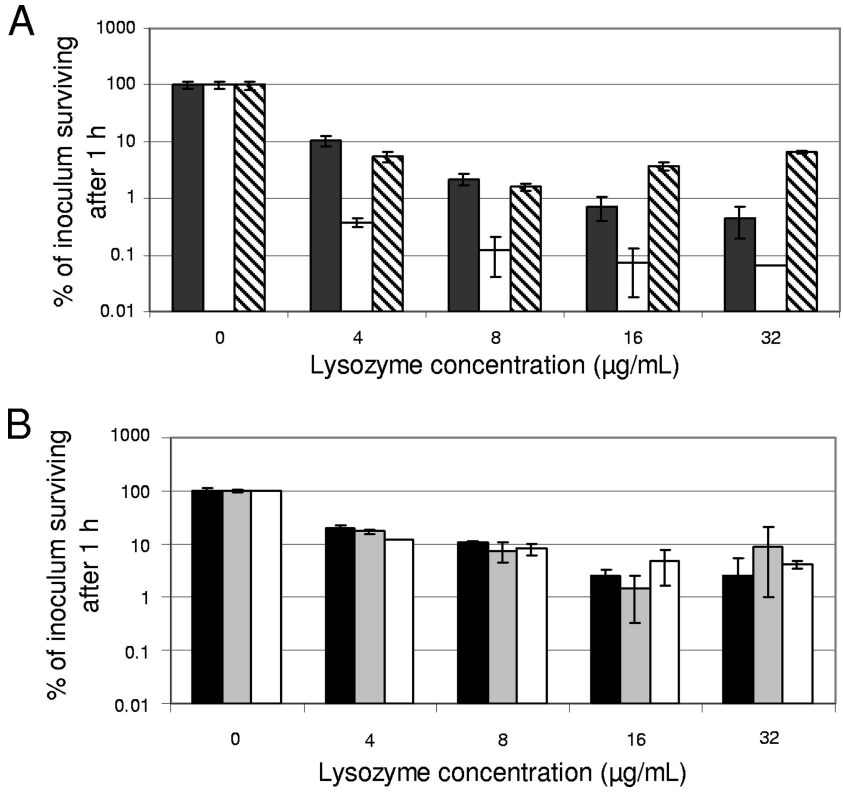

FIG. 1. (A) Contribution of *per* to lysozyme resistance in EPEC strain E2348/69. Percentages of inocula surviving after incubation with increasing concentrations of lysozyme for 1 h are shown. Each data point is the mean of the results of three experiments, and error bars represent standard deviations. Test strains are wild-type E2348/69 (black bars); OG127, the isogenic *per* mutant (unshaded bars); and the *per* mutant carrying the *perABC* genes cloned into pBR322(pINKper31) (hatched bars). Differences between E2348/69 and OG127 were significant at 4, 8, and 16 μg/ml of lysozyme ($P < 0.05$) (B) Lysozyme resistance in type III secretion- and bundle-forming pilus-deficient mutants. Mean percentages of inocula surviving after incubation with increasing concentrations of lysozyme are shown. Error bars represent standard deviations. Test strains are wild-type E2348/69 (black bars); CVD452, which is unable to effect type III secretion due to a deletion of structural protein *escN* (gray bars); and UMD901, with a deletion of *bfpA*, the structural subunit of bundle-forming pili (white bars).

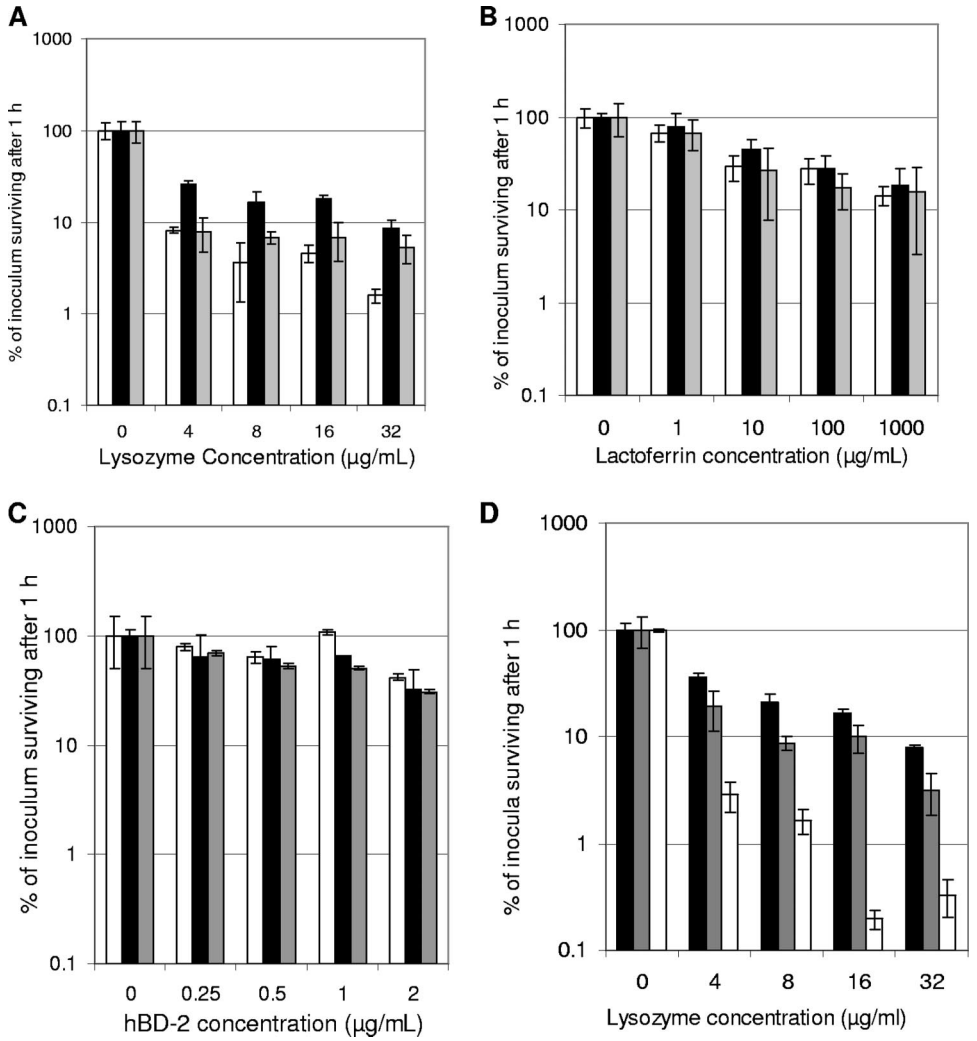

FIG. 2. (A to C) Resistance of *E. coli* K12 strain DH5α (unshaded bars) with and without the *espC*-containing plasmid pJLM174 to lysozyme (A), lactoferrin (B), and human β-defensin 2 (hBD-2) (C). Transcription of *espC* from this pBAD construct is induced by 0.2% arabinose (black bars) and repressed by 2% glucose (gray bars). Differences seen between DH5α alone and DH5α(pJLM174) with *espC* turned off by glucose were not significant, whereas differences between DH5α alone and DH5α(pJLM174) with *espC* induced by arabinose were significant at 8 μg/ml ($P = 0.03$) and highly significant at all other concentrations tested ($P < 0.004$). (D) Lysozyme resistance in wild-type *E. coli* strain HB101 (white bars) and in HB101 carrying the cloned *per* genes in plasmid pINKper31 (black bars) or a vector control (gray bars). Differences between HB101(pINKper31) and HB101(pBR322) were significant at 4, 16, and 32 μg/ml of lysozyme ($P < 0.05$).

able to demonstrate that EspC expression in strain DH5α resulted in an up-to-20% increase in survival upon lysozyme challenge (Fig. 2A). The magnitude of resistance conferred by EspC was significant at all test concentrations ($P < 0.05$) but was less pronounced than the differential between the resistance of E2348/69 and that of its *per* mutant (Fig. 1b). Therefore, *espC* may account for some lysozyme resistance in EPEC strain E2348/69, but probably not all of it. Furthermore, strain B171, an EPEC strain that is *espC* negative, is more resistant than K-12 but less resistant than E2348/69 (Table 1). As shown in Fig. 2B and C, in contrast to the resistance to lysozyme, *espC* did not confer resistance to lactoferrin or human β-defensin 2 in peptide sensitivity experiments. We therefore concluded that EspC-mediated resistance was due to a specific antilysozyme mechanism, as opposed to a generalized peptide resistance mechanism.

Like related serine protease autotransporters of the *Enterobacteriacaeae* (SPATEs), EspC has a GDSGS motif and cleaves multiple substrates, including spectrin and hemoglobin (9). Lysozyme does not possess any Arg-Arg motifs, a known cleavage site for EspC (9), but it is possible that there are other, as-yet-unknown EspC target sites. Lys-Arg motifs at positions 13 and 14 and 97 and 98, for example, would be candidate cleavage sites (Fig. 3A). Targeted proteolytic degradation is an antimicrobial-peptide-resistance mechanism that has been documented in a number of organisms, ranging from *Staphylococcus aureus* and *Proteus mirabilis* to *Leishmania major* (4, 15, 16, 22). We therefore sought to determine whether lysozyme was cleaved after incubation with DH5α(pJLM174) and arabinose, conditions under which EspC cleaves spectrin (data not shown) and also confers lysozyme resistance.

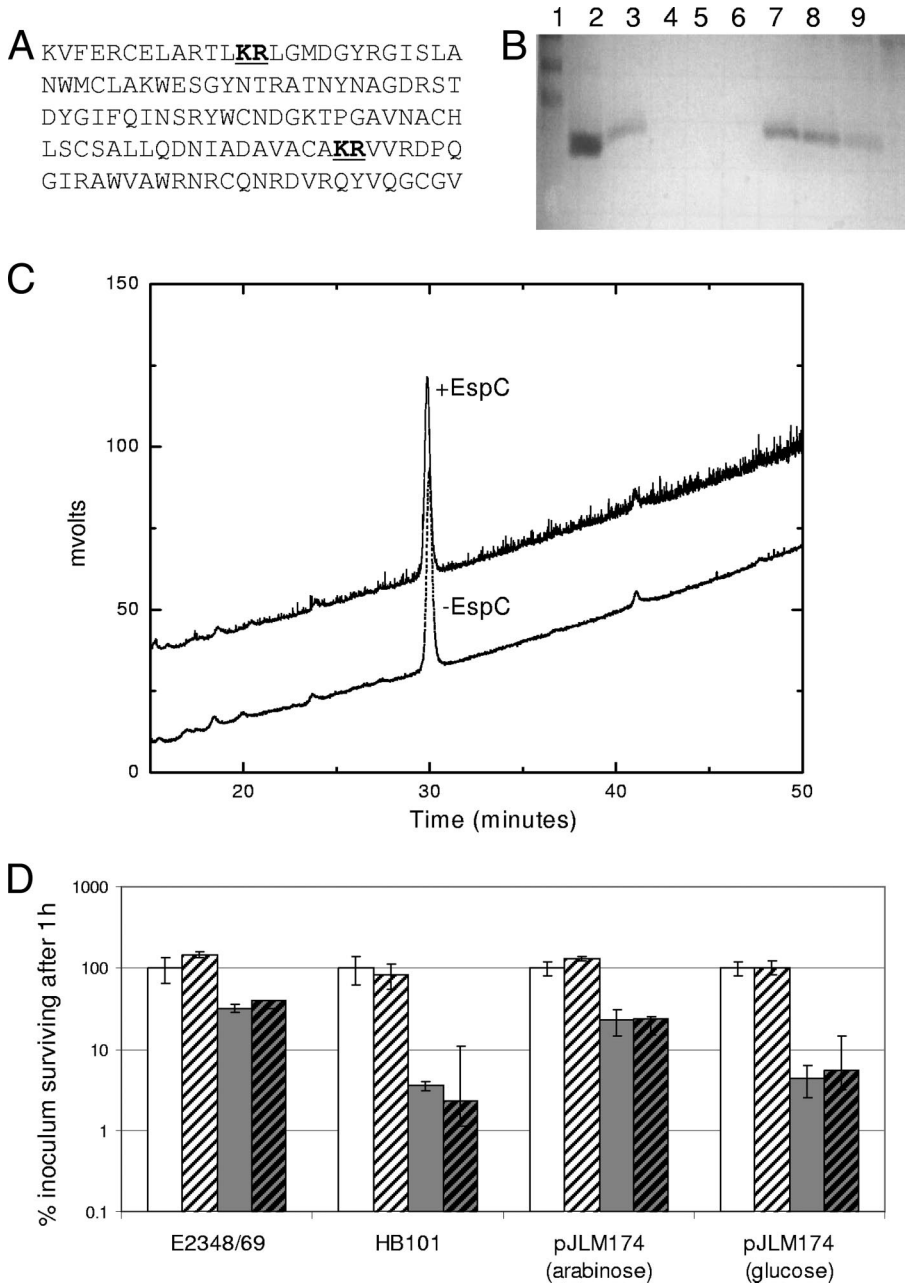

FIG. 3. (A) Sequence of lysozyme with adjacent basic residues, hypothetical EspC target cleavage sites, underlined and in boldface. Lysozyme was incubated with DH5α carrying the *espC* expression clone pJLM174 activated with arabinose or repressed with glucose and then analyzed by SDS-PAGE and RP-HPLC. (B) Coomassie blue-stained SDS-PAGE gel of culture supernatants of DH5α without (lanes 4 and 7) and with the *espC*-containing plasmid pJLM174 induced with 2% arabinose (lanes 5 and 8) or repressed with 2% glucose (lanes 6 and 9). Lanes 7 to 9 contain 16 μg/ml lysozyme. Lane 1, molecular-weight marker; lane 2, 32 μg/ml lysozyme; and lane 3, 64 μg/ml lysozyme incubated with medium control. (C) Results of RP-HPLC of lysozyme (16 μg/ml) incubated with pJLM174 induced with 2% arabinose or repressed with 2% glucose. The retention time for the sample with *espC* turned off (−EspC) was 29.94 min, or 44.94% acetonitrile, while that for the sample with *espC* turned on (+EspC) was 29.86 min, or 44.86% acetonitrile. An offset has been applied for clarity. (D) Survival of test strains after untreated incubation (unshaded bars) or incubation with 4 μg/ml of lysozyme (shaded bars) in the presence (respective hatched bars) and absence of serine protease inhibitor 2 mM PMSF. Error bars represent standard deviations.

Lysozyme intactness was assessed after peptide sensitivity experiments (7) performed in 3-ml reaction tubes. Following incubation at 37°C for 1 h under test conditions, an aliquot was removed for viable-cell counting and bacterial cells were removed from the rest of the suspension by centrifugation and filtration. A 2-ml amount of the supernatant was transferred onto a YM-10 Centricon filter device (Millipore) and concentrated to 1/10 of the original volume according to the manufacturer's instructions. Retentates were analyzed by sodium dodecyl sulfate-polyacrylamide gel elec-

trophoresis (SDS-PAGE) and Coomassie staining and by reversed-phase high-pressure liquid chromatography (RP-HPLC). RP-HPLC was performed on a 250- by 4.6-mm analytical Varian Microsorb 100-5 C_{18} column using a gradient of 20 to 70% acetonitrile. The mobile phase contained water–acetonitrile–0.1% trifluoracetic acid.

Lysozyme exposed to induced and uninduced *E. coli* DH5α (pJLM174) did not show any sign of degradation as detected by both SDS-PAGE and RP-HPLC (Fig. 3B and C). To rule out the possibility that cleavage was occurring close to the end of the protein or that EspC was cleaving another factor which then conferred resistance, we measured the survival of induced and uninduced DH5α(pJLM174) in medium containing phenylmethylsulphonyl fluoride (PMSF), which has previously been shown to inhibit EspC serine-protease activity. We found that 2 mM PMSF, which obliterates the ability of EspC to cleave spectrin, did not interfere with EspC-mediated lysozyme resistance ($P > 0.05$) (Fig. 3D).

Another SPATE, Pic from enteroaggregative *E. coli*, does not cleave lysozyme, and although there is no evidence that it confers lysozyme resistance, its mucinolytic activity is in part attributed to lectin-like properties (13). We hypothesized that EspC might physically interact with lysozyme in a manner similar to the K-12 lysozyme inhibitor Ivy (1). We incubated bacterial supernatants of EPEC strain E2348/69 and of induced and uninduced *E. coli* DH5α(pJLM174), as well as of DH5α, with biotinylated lysozyme (Sigma). Although we were able to detect both lysozyme and EspC by Western blotting at the end of incubation, EspC could not be magnetically copurified with biotinylated lysozyme, using μMACS streptavidin microbeads (Miltenyi biotec, Auburn, CA) on a column separator in accordance with the manufacturer's protocols (data not shown). Therefore, at least under test conditions, we were unable to demonstrate such an interaction and the mechanism of EspC-mediated lysozyme resistance remains unknown.

Recently, it was demonstrated that although EspC is efficiently autotransported from the bacterial cell, the EPEC TTSS is needed for host cell internalization (27). As there is a time lag for internalization, we speculate that the intracellular activities of EspC are important during long-term colonization, such as in persistent diarrhea. Although EspC-positive EPEC are commonly associated with outbreaks, a role for the protein in early stages of infection, such as adherence, invasion, or signal transduction, has not been found (23). Resistance to lysozyme may be important in establishing infection and may, along with EspC enterotoxin activity, contribute significantly to acute disease.

espC confers supplementary lysozyme resistance on nonpathogenic *E. coli* but is unlikely to account for all the *per*-mediated resistance in EPEC strain E2348/69, nor can it explain the supplementary lysozyme resistance seen in *espC*-negative EPEC strain B171 (Fig. 1A). Since we have ruled out many of the known virulence factors shared by B171 and E2348/69, we hypothesized that Per-regulated core *E. coli* genes could contribute to lysozyme resistance. We measured resistance in the nonpathogenic *E. coli* strain HB101 bearing the *perABC* clone pINKper31 in comparison to that of the same strain carrying the vector alone. As shown in Fig. 2D, the pINKper31 *per* clone conferred significant levels of resistance on HB101 at 4, 16, and 32 μg/ml of lysozyme ($P < 0.05$).

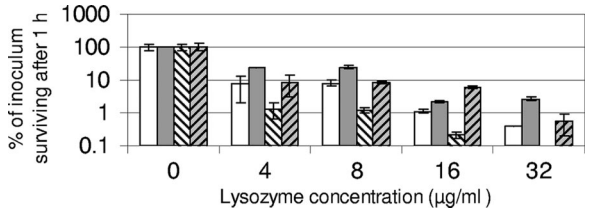

FIG. 4. Survival of MG1655 with deletion of *tolB* (unhatched bars) or *tolB* and *ivy* (hatched bars) carrying a *per* clone, pINKper31 (gray bars), or the vector plasmid (white bars) after treatment with lysozyme for 1 h. Error bars represent standard deviations, and differences between data from strains carrying pINKper31 and from those carrying pBR322 were significant ($P < 0.05$).

Interestingly, the observed impact of this second effect was greater at higher concentrations of lysozyme, where *espC* conferred a less-significant protective effect (Fig. 2A).

Recently, Abergel et al. reported the crystal structure of Ivy, an *E. coli* protein that binds to the active site of lysozyme, and identified orthologues in other species (1). We sought to determine whether upregulation of Ivy-mediated protection could account for the resistance conferred by Per in *E. coli* K-12. Isogenic derivatives of *E. coli* K-12 strain MG1655 (Δ*tolB*) and (Δ*tolB* Δ*ivy*) were obtained from C. Abergel. We recovered 1 log more Δ*tolB* bacteria than Δ*tolB* Δ*ivy* bacteria in our assay following treatment with concentrations ranging between 4 and 32 μl of lysozyme, consistent with the findings of Abergel et al. (1). Similar results were documented when the pBR322 vector was transformed into both strains (Fig. 4). However, *perABC* genes cloned into this vector conferred a significant increase in lysozyme resistance in both the Δ*tolB* and Δ*tolB* Δ*ivy* strains ($P < 0.05$). Therefore, a factor(s) other than *ivy* must account for this resistance.

Commensal *E. coli* bacteria possess genes encoding an inhibitor of vertebrate lysozyme, Ivy, as well as a recently described *m*embrane-bound *l*ysozyme *i*nhibitor (MliC) (1). Supplementary to these mechanisms, EPEC strains have multiple, distinct, and Per-activated lysozyme resistance mechanisms. One of these mechanisms involves the activation of an EPEC-specific SPATE protein, *espC*, and the other involves one or more factors present in nonpathogenic *E. coli* K-12. The *ivy* gene is present in K-12 but is not activated by Per. Instead, at least one other as-yet-unidentified factor, possibly MliC, contributes to *per*-mediated lysozyme resistance in EPEC. Many pathogens resist human antimicrobial peptides, but to our knowledge, this is the first report of antimicrobial-peptide resistance in EPEC. The continued characterization of antimicrobial-peptide resistance could better inform the epidemiology of this important pathogen, as well as improve the understanding of and response to resistance to natural and artificial antimicrobials.

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