Contribution of BlaA and BlaB β -Lactamases to Antibiotic Susceptibility of *Yersinia enterocolitica* Biovar 1B[∇]

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Highly pathogenic *Yersinia enterocolitica* biovar 1B produces two distinct β -lactamases, BlaA and BlaB. Mutants of a representative biovar 1B isolate were constructed and evaluated to determine the extent of limitation of susceptibility to broad-spectrum β -lactam antibiotics by BlaA and BlaB. The results demonstrated that BlaA, a class A enzyme, plays a significant role in limiting susceptibility to penicillins and cephalosporins. The contribution of BlaB, a class C enzyme, was less profound and was limited primarily to cephalosporin susceptibility.

Yersinia enterocolitica is a human gastrointestinal pathogen with a worldwide distribution. Infection results from consuming contaminated food or water and generally leads to a self-limiting gastroenteritis that principally causes abdominal pain and diarrhea in otherwise healthy individuals (3). The majority of infections occur in young children and infants, however, in whom this gastroenteritis can lead to a potentially fatal systemic infection that has a mortality rate approaching 50% (3, 7). The species *Y. enterocolitica* is fairly heterogeneous, with six distinct biovars (1A, 1B, 2, 3, 4, and 5) based on pathogenicity, geographic distribution, and ecological niche (1). It is well established that resistance to β -lactam antibiotics is common among *Y. enterocolitica* strains but that the level and spectrum of resistance are variable due to the differential expression and activities of two different β -lactamases, BlaA and BlaB (4, 9–11, 16, 18). Previous studies have documented the distribution of these β -lactamases in a wide variety of clinical and environmental *Y. enterocolitica* isolates and have determined the overall resistance or susceptibility of these strains to various β -lactam antibiotics (9–11, 13, 16, 18, 19). It appears that strains within a given biovar tend to display similar phenotypes (18).

This study focused solely on the highly pathogenic strain 8081 of *Y. enterocolitica* biovar 1B, which causes severe gastroenteritis in humans. Biovar 1B strains contain an assortment of genes encoding virulence factors responsible for increased pathogenesis compared to that of other biovars of *Y. enterocolitica* (5, 20). Like infections caused by most other biovars of *Y. enterocolitica*, biovar 1B infections are not easily treatable with β -lactam antibiotics, because these strains carry genes encoding two distinct β -lactamases. BlaA is a class A enzyme, and BlaB (AmpC) is a class C enzyme, as defined by their homologies to other β -lactamases. This leads to the assumption that BlaA is an enzyme primarily acting on penicillins, whereas BlaB is more specific to cephalosporins (2, 11, 14, 15). These observations are supported by a limited number of experi-

ments that examined the effects of specific inhibitors on the β -lactamase activities of cellular lysates derived from various *Y. enterocolitica* clinical isolates (11, 18, 19). The goal of this study was to test this hypothesis for the well-defined and -studied strain *Y. enterocolitica* 8081. The approach was to examine the antibiotic susceptibilities of isogenic strains derived from *Y. enterocolitica* 8081 that were defective in the production of one or both β -lactamases (BlaA and/or BlaB).

Examination of the genomic sequence of *Y. enterocolitica* 8081 revealed that BlaA and BlaB are encoded by open reading frames (ORFs) YE2019 and YE2440, respectively. These open reading frames were correspondingly renamed *blaA* and *blaB*. To examine whether the loss of these β -lactamases individually would have an effect on antibiotic susceptibility, *blaA* and *blaB* deletion mutations were constructed and introduced by allelic exchange into strain JB580v, an isogenic derivative of *Y. enterocolitica* 8081 defective for a host DNA restriction system (6, 12). Each deletion allele, which removed the corresponding ORF of the gene, was generated using the four-primer overlap extension method of PCR (21). The allele was cloned into a plasmid derived from pMRS101 that was subsequently used for the selection and screening procedure to isolate mutants arising from allelic exchange (12). Each mutant strain genotype was confirmed by a diagnostic PCR-based procedure. This collection of mutants was then characterized to establish that they were appropriately deficient in the production of either BlaA or BlaB (Fig. 1). β -Lactamase detection following protein fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a method adapted from Sharma et al. using nitrocefin as a chromogenic substrate (8, 17). Hydrolysis of nitrocefin by a β -lactamase results in the formation of a red product, thereby revealing the apparent location of each β -lactamase (Fig. 1). Strain JB580v produced proteins with the expected molecular masses of BlaA and BlaB at 31.6 kDa and 43.3 kDa, respectively. In contrast, for each of the deletion mutants (GY5717 [*blaA*], GY5542 [*blaB*], and GY5718 [*blaA blaB*]), the results indicated that the protein corresponding to BlaA and/or BlaB was absent as expected. Furthermore, we noted that cultures exposed to subinhibitory concentrations of imipenem appeared to produce greater amounts of both BlaA and BlaB. This is consistent with the findings of previous studies and

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FIG. 1. *Y. enterocolitica* biovar 1B produces BlaA and BlaB β-lactamases. Each strain was grown in TYE broth under inducing (+ imipenem) and noninducing (– imipenem) conditions. A total-protein extract from each sample was subjected to SDS-PAGE. The acrylamide gel was washed to renature proteins. The location of each active β-lactamase was visualized in an impregnated gel with subsequent hydrolysis of the chromogenic substrate nitrocefin. The molecular mass of each β-lactamase (31.6 kDa for BlaA; 43.3 kDa for BlaB) was estimated by comparison to protein mass standards.

demonstrates that the production of these enzymes is inducible. Collectively, these data confirmed that *Y. enterocolitica* 8081 and the various deletion derivatives were suitable for further analysis.

In previous studies, the inferred contribution of BlaA and BlaB to limiting the susceptibility of *Y. enterocolitica* to penicillins and cephalosporins was based largely on the measurement of enzymatic activity from cell lysates. Our new collection of β-lactamase mutants allowed for this approach to be systematically evaluated and challenged (8, 10). Compared to those from JB580v, cell lysates from strain GY5718 ($\Delta blaA \Delta blaB$) exhibited a 10-fold reduction in activity. Strains GY5717 ($\Delta blaA$) and GY5542 ($\Delta blaB$) similarly displayed intermediate levels of β-lactamase activity (Fig. 2). The presence of imipenem in the growth medium resulted in a measurable increase in the level of β-lactamase activity from BlaB, further supporting the idea that production of these enzymes is subject to regulatory control. From the analysis of the mutant collection using the enzymatic measurement approach, it appeared that both BlaA and BlaB were likely to play important roles in decreasing the susceptibility of *Y. enterocolitica* to a variety of β-lactam antibiotics (Fig. 2). However, we wanted to challenge

FIG. 2. BlaA and BlaB contribute to the overall ability of *Y. enterocolitica* biovar 1B to hydrolyze the commonly used β-lactam substrate nitrocefin. All strains were examined following cultivation in TYE medium without (–) or with (+) the addition of +0.1 mg/ml imipenem. Bacteria were collected from each culture, washed, and assayed by the method of O’Callaghan et al. (8). Enzyme activity is expressed as micromoles of nitrocefin hydrolyzed per minute per milligram of protein; the calculation was based on the extinction coefficient of 20,500 M^{–1} cm^{–1} as published by the manufacturer (Fisher).

TABLE 1. Resistance of *Y. enterocolitica* to different β-lactam antibiotics^a

Antibiotic	MIC (μg/ml) for strains with the following genotype:			
	WT ^b	$\Delta blaA$	$\Delta blaB$	$\Delta blaA \Delta blaB$
Penicillins				
Ampicillin	4	0.5	4	0.19
Penicillin G	24	4	24	1
Amoxicillin	24	1.5	24	0.38
Piperacillin	0.75	0.19	0.75	0.094
Cephalosporins				
Cefotaxime	0.023	0.023	0.016	0.012
Cefuroxime	0.75	0.5	0.75	0.5
Ceftriaxone	0.023	0.016	0.016	0.012
Carbapenem (imipenem)	0.25	0.25	0.25	0.25

^a Assays were completed in quadruplicate on solidified Mueller-Hinton medium, and incubation was carried out at 35°C for 20 h in accordance with the recommendations of the Etest strip manufacturer (bioMérieux).

^b WT, wild type.

this common inference by examining how these results translate into antibiotic susceptibility within a biological context.

To gain insight into the roles of BlaA and BlaB in the survival of bacteria when challenged with antibiotics, we completed MIC assays using standardized Etest strips for a variety of penicillins, cephalosporins, and the carbapenem imipenem (Table 1). This collection of antibiotics overlaps with those that were previously tested in other studies (11, 18, 19). The $\Delta blaA$ strain had a significantly lower MIC than either the wild-type strain or the $\Delta blaB$ strain, both of which had the same MIC in each case. Interestingly, it appears that BlaB is epistatic to BlaA, since the double mutant had a reproducibly lower MIC than either strain carrying a mutation affecting the production of a single β-lactamase. In the case of cephalosporins, neither β-lactamase was dominant in providing resistance. There was a minimal difference between wild-type and mutant MIC values, but there seemed to be a synergistic effect, with the double mutant having a lower MIC than either single mutant. Neither β-lactamase played any role in resistance to imipenem.

The study of an isogenic collection of *Y. enterocolitica* mutant strains revealed both expected and unexpected results. It was not surprising that BlaA, a class A β-lactamase, was important for limiting susceptibility to penicillins. However, the hypothesis that loss of BlaB (AmpC), a class C β-lactamase, would contribute significantly to increased susceptibility to cephalosporins failed. This highlights the value of examining the specific roles of β-lactamase within a biological context. Initial assessments using *in vitro* assays for enzyme activities are often considered to be robust surrogate assays for defining antibiotic resistance but should be considered presumptive rather than conclusive. In the case of *Y. enterocolitica* 8081, it is BlaA that contributes most to the wide spectrum of antibiotic resistance. It will be interesting to examine other *Y. enterocolitica* isolates in order to extend this analysis and to further evaluate if other isoforms of BlaA and BlaB differentially contribute to antibiotic susceptibility.

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