

ANTIMICROBIAL RESISTANCE

Expression of β -lactamases in *Yersinia enterocolitica* strains of biovars 2, 4 and 5

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Characteristic patterns of susceptibility to β -lactam antibiotics are associated with different biovars of *Yersinia enterocolitica*. To elucidate the basis for these differences, the β -lactamases of strains of *Y. enterocolitica* biovars 4 (n = 63), 2 (n = 12) and 5 (n = 10) were characterised. PCR fragments were generated from the β -lactamase A (*blaA*) and B (*blaB*) genes; in addition, β -lactamase induction tests were performed with imipenem as the inducer and β -lactamase inhibition assays were undertaken with aztreonam and clavulanic acid. All the strains yielded PCR amplification fragments with primers to *blaA* and *blaB*. Biovar 4 strains had uniform patterns of β -lactamase induction and inhibition: uninduced biovar 4 strains predominantly expressed BlaA, but low-level expression of BlaB was also detected; after induction, biovar 4 strains predominantly produced BlaB. β -Lactamase expression varied between and within biovars 2 and 5: uninduced strains predominantly expressed either BlaA or BlaB, or exclusively BlaB; after induction BlaB was predominantly or exclusively expressed. Both the basal and induced levels of β -lactamase varied within biovars 2 and 5. Some biovar 5 strains were not inducible; these predominantly produced BlaA. The results of this study show that biovar 2, 4 and 5 strains contain both *blaA* and *blaB*, but that the expression of the enzymes is regulated differently between the biovars, and varies within biovars 2 and 5. There was some correlation between antibiogram and the clusters defined from the β -lactamase induction and inhibition tests, but it was not possible to predict β -lactamase expression profiles from MIC data.

Introduction

Yersinia enterocolitica is an important human pathogen, associated with enterocolitis, lymphadenitis and ileitis. The species is divided into six biovars (1A, 1B, 2, 3, 4 and 5) [1, 2], but atypical strains also exist. The biovars differ in their geographical distributions, ecological niches and pathogenic properties. This study examined strains of biovar 4, 2 and 5.

Biovar 4 (serovar O3) is predominant in clinical specimens world-wide. Biovar 2 strains of serovar O5,27 are mainly isolated in the USA and Japan, where they are associated with human yersiniosis. Human infections with biovar 2 strains are not uncommon in Europe, but are caused by members of serovar O9. The natural reservoir of both biovars 2 and 4 is the pig. Biovar 5 strains (serovars O2 and 3) are also pathogenic for man, but the clinical significance of

this biovar, which has a natural reservoir in hares, is not well understood.

Previous studies that aimed to correlate antibiotic susceptibility patterns with biovar in *Y. enterocolitica* have yielded conflicting results. Stolk-Engelaar *et al.* examined 335 strains and could not associate susceptibility patterns with biovar or serovar [3], but others have found relationships [4–9]. Previous studies at this laboratory confirmed the latter view, finding a relationship between biovar and susceptibility to ticarcillin, amoxycillin-clavulanate, cefazolin, loracarbef, cefoxitin, cefpodoxime, cefixime and other β -lactam antibiotics (Table 1 and [10]). Biovar-specific susceptibilities to β -lactam agents may relate to the regulation and expression of β -lactamases. As long ago as 1975, Cornelis and Abraham described two chromosomally encoded β -lactamases, BlaA and BlaB, in *Y. enterocolitica* [11]. BlaA is a constitutively expressed class A enzyme related to those of *Klebsiella oxytoca* and *Proteus vulgaris* [12, 13]. It is completely inhibited by clavulanic acid (20 μ M), but not by aztreonam [14]. BlaB is an inducible class C enzyme related to the AmpC β -lactamases of other Enterobac-

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Table 1. Susceptibility of *Y. enterocolitica* biovars 2, 4 and 5 to antibiotics (from reference [10])

Biovar	Number of isolates	Phenotypic group	Number of isolates	MIC range (mg/L)				Range of ratio between amoxycillin: amoxycillin-clavulanate MICs
				cefixime	cefepodoxime	ticarclillin	amoxycillin-clavulanate	
4	63	4-0*	63	0.06–0.5	0.25–1	64–256	0.5–4	4–7
2	12	2-I	10	0.5–2	1–2†	64–256	8–32	1–3
		2-II	2	2–4	2–4	4	8–16	2–3
		5-I	4	0.125	0.25	64–128	0.5–2	5–6
5	10	5-II	3	≤ 0.06	0.125	32–64	0.125	8
		5-III	1	4	4	256	16	3
		5-IV	2	< 0.06	0.06	0.25–0.5	0.5	1–2

*One susceptibility pattern to these antibiotics was detected.

†One strain had an MIC of 0.5, one an MIC of 4 mg/L to cefepodoxime.

teriaceae [15]; it is inhibited by aztreonam (10 µM) and cloxacillin (10 µM), but not by clavulanic acid [14]. The nucleotide sequences of *blaA* and *blaB* genes have been published [12, 15].

To elucidate the molecular basis for the differences in susceptibilities to β-lactam antibiotics of biovar 2, 4 and 5 strains, their β-lactamases were characterised genotypically and phenotypically.

Material and methods

Bacterial strains

A total of 85 *Y. enterocolitica* strains belonging to biovars 4 (n = 63), 2 (n = 12) and 5 (n = 10) were included; most were isolated from clinical specimens or mammals in Germany. All the biovar 4 strains originated from the University Hospital of Bonn, Germany or from different hospital patients and outpatients in cities in Southern Germany. Biovar 2 strains were isolated from clinical specimens (n = 7), raw milk (n = 2), the intestines of dead pigs (n = 2) and water (n = 1). Strains of biovar 5 were predominantly isolated from hares (n = 7), but also from clinical specimens (n = 3). Hare-associated biovar 5 strains and two biovar 2 strains were used as reference strains for these biovars; these strains were kindly provided by S. Aleksic (Hamburg, Germany) and had been biotyped and serotyped at the Hygiene Institute of Hamburg. A reference strain of biovar 4 and strains of other *Yersinia* spp. (see *Results*) were kindly provided by J. Heesemann (Munich, Germany). The clinical isolates were non-replicates, all from different patients on different wards.

PCR for *blaA* and *blaB*

All the strains were examined by PCR for the presence of *blaA* and *blaB*. Template DNA was obtained as described by Nakajima *et al.* [16], but without the initial boiling and final centrifugation. The primer pair used to amplify a 439-bp fragment of *blaA* were *blaA*5 (⁴²¹AAATGCGCTACCGGCTTCAG⁴⁴⁰) and *blaA*3 (⁸⁹⁸AGTGGTGGTATCACGTGGGT⁸⁷⁹); those used to amplify a 781-bp fragment of *blaB* were *blaB*5

(¹⁶⁰¹CCCACTTTATACCTTGGCACAAA¹⁶²³) and *blaB*3 (²⁴²⁶GAACATATCTCCTGCCTGGAAAT²⁴⁰⁴). Each PCR was performed in a volume of 100 µl, containing 10 pmol of each of the four primers, 4 nmol of each deoxynucleotide triphosphate and 5 U *Taq*-DNA-polymerase (Promega, Heidelberg, Germany) in the enzyme buffer supplied by the manufacturer, also containing 0.75 mM MgCl₂. PCR temperature profiles were as follows: one initial denaturation step at 95°C for 5 min, 25 cycles of 30 s at 50°C, 60 s at 72°C, 30 s at 95°C, followed by one final cycle (50°C 30 s, 72°C 5 min). The PCR products were electrophoresed on agarose 1.5% gels, stained with ethidium bromide and photographed under UV illumination.

Measurement of β-lactamase induction

Thirteen strains of biovar 4 and all the biovar 2 and 5 strains were tested by a modification of the induction test described by Pham and Bell [17]. Briefly, an overnight (16 h) culture grown on IsoSensitest Agar (Oxoid) at 28°C was used to prepare a saline suspension with an OD₆₄₀ of 0.5 (Hitachi 150-200 Spectrophotometer). Two-ml amounts of the suspensions were added to each of two 100-ml Erlenmeyer flasks, each containing 18 ml of Tryptone Soya Broth (Merck, Darmstadt, Germany) which had been pre-warmed to 28°C. These cultures were incubated at 28°C in a water bath with shaking at 100 rpm until the growth had achieved an OD₆₄₀ of 0.2 ± 0.02. Imipenem was then added to one of the flasks to a final concentration of 0.5 mg/L. Phenotypic group 5-II strains did not tolerate this concentration and the imipenem concentration was reduced to 0.125 mg/L. No inducer was added to the other flask, which served as a control. Incubation of both flasks was allowed to continue in a water bath with shaking for 2.5 h. Two-ml volumes from each flask were withdrawn and used to measure the absorbance and for a viable cell count by spread plates; a further 10-ml volume was centrifuged (15 min, 5000 g) and the pellet was resuspended in 1 ml of 0.05 M phosphate buffer, pH 7.0. Sonication on ice (Sonifier B12, Branson Sonic Power, Danbury) and centrifugation for 60 min at 3000 g yielded a crude supernate for β-lactamase assays. β-Lactamase activity

was quantified as described by Peter *et al.* [18] with nitrocefin (50 μM) as the substrate [19]. The protein content of each sample was determined by the method of Lowry *et al.* [20], with bovine serum albumin as the standard.

Measurement of β-lactamase inhibition

The inhibition of β-lactamases was measured according to Pham *et al.* [14] with some modifications. Crude enzyme extracts were diluted in phosphate-buffered saline, pH 7.0, to yield a β-lactamase activity of 500–1000 μmol nitrocefin hydrolysed/min/L. Fifty μl of the diluted extract were added to 850 μl of 0.05M potassium phosphate (pH 7.0), followed by 50 μl of either clavulanic acid (2 mg/L in water), aztreonam (2 mg/L in water) or water (control). In some cases these inhibition tests were conducted with 1 and 20 mg of the inhibitors/L. The tubes were rocked, then allowed to stand at 28°C for 15 min, after which 50 μl of 50 μM nitrocefin were added. The percentage inhibition was calculated with the formula described by Jimenez-Valera *et al.* [21], modified by calculating the rate of change of absorptions per minute (ΔAbs/min) as a proxy for activity.

Results

PCR for *blaA* and *blaB*

Genes for both *BlaA* and *BlaB* were found in all the strains. This is illustrated for biovar 4 strains in Fig. 1. No bands corresponding to *blaA* and *blaB* were obtained from other *Yersinia* spp. (*Y. pestis*, *Y. pseudotuberculosis*, *Y. mollaretii*, *Y. kristensenii*, *Y. frederiksenii*, *Y. ruckeri*) nor from other Enterobacteriaceae (*Escherichia coli*, *Shigella sonnei*, *E. hermannii*, *K. oxytoca*, *K. pneumoniae*, *Citrobacter freundii*, *C. koseri*, *C. amalonaticus*, *Enterobacter cloacae*, *Serratia marcescens*, *Morganella morganii*, *Proteus vulgaris*, *P. mirabilis* and *Salmonella enterica*) (not shown).

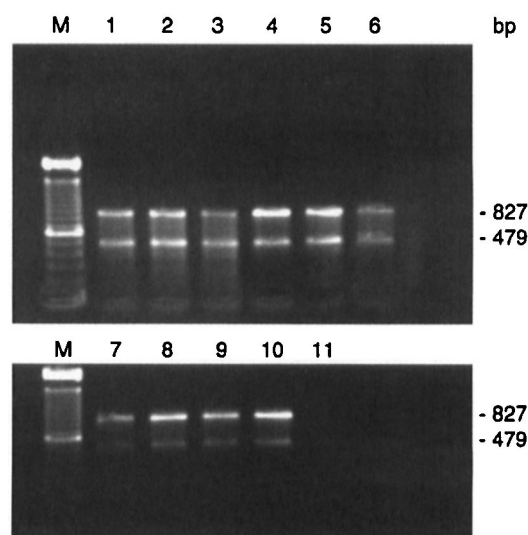


Fig. 1. PCR products from 10 *Y. enterocolitica* biovar 4 strains: the 479-bp bands indicate the presence of *blaA*, the 827-bp bands the presence of *blaB*. Lane M, 100-bp ladder DNA marker; lanes 1–10, representative strains of biovar 4 (1, strain 54-15; 2, strain 54-16; 3, strain 54-17; 4, strain 54-18; 5, strain 54-22; 6, strain 54-23; 7, strain 54-30; 8, strain 54-35; 9, strain 54-37; 10, strain 54-38); 11, control (water).

Template DNA prepared by simple boiling was not appropriate for PCR with *Y. enterocolitica*; because of the presence of heat-stable deoxyribonucleases produced from several strains of *Y. enterocolitica*, PCR products were not obtained or were degraded during overnight storage at 4°C. Prior treatment of the template DNA with proteinase K allowed PCR products to be obtained, although not all were detectable after overnight storage at 4°C.

β-Lactamase induction and inhibition

A summary of induction and inhibition results is shown in Table 2. The characteristic inhibition profiles of *BlaA* and *BlaB* allowed interpretation of the inhibition

Table 2. β-Lactamase induction and inhibition with *Y. enterocolitica* biovars 4, 2 and 5

Phenotypic group*	Cluster†	Number of strains	β-Lactamase induction			β-Lactamase inhibition (%)			
			Specific activity (μmol/min/mg)			Uninduced, in presence of		Induced, in presence of	
			Uninduced	Induced	Ratio	AZT‡	CLA‡	AZT‡	CLA‡
4-0	nd	13	0.83–1.3	1.4–2.9	1.6–3.1	15–20	80–85	55–80	20–45
2-I	A ₁	1	7.4	17	2.3	90	10	100	0
	A ₂	3	0.78–0.93	1.9–3.1	2.4–3.3	40	60	80–90	10–20
	A ₃	1	0.65	17	26	25	75	> 95	< 5
	B	5	0.11–0.17	5.4–14	36–93	100	0	100	0
2-II	nd	2	0.5–1.0	7.6–13	13–15	100	0	100	0
5-I	A	3	0.42–0.76	1.6–3.0	2.2–4.4	25–30	70–75	60–85	15–40
	B	1	0.19	0.21	1.1	10	90	10	90
5-II	nd	3	0.23–0.29	0.28–0.31	1.0–1.2	5–15	85–95	5–15	85–95
5-III	nd	1	0.36	20	56	75	25	90	10
5-IV	nd	2	0.04–0.08	1.1–1.4	14–33	100	0	100	0

nd, no difference.

*According to reference [10].

†According to data obtained from induction and inhibition tests.

‡2 mg/L.

results as follows: where clavulanate inhibited activity completely, BlaA was inferred to be the only β -lactamase present; where aztreonam inhibited activity completely, BlaB was the only enzyme; where clavulanate inhibited most of the activity, BlaA was the predominant enzyme and where aztreonam inhibited most of the activity, BlaB was deduced to be predominant. Biovar 4 strains showed a uniform pattern of β -lactamase induction and inhibition. An increase in β -lactamase activity, ranging from 1.6- to 3.1-fold, was observed in all biovar 4 strains after induction. Inhibition tests revealed that uninduced biovar 4 strains expressed predominantly BlaA and to a lesser extent BlaB (i.e., inhibition was seen with clavulanate, less so with aztreonam). After induction, this pattern was reversed, indicating that the strains predominantly produced BlaB. β -Lactamase expression differed between and within biovars 2 and 5: uninduced biovar 2 and 5 strains predominantly expressed BlaA, predominantly BlaB or exclusively BlaB, but never BlaA alone. Both the basal levels of β -lactamase expression and the increase of activity after induction differed between different strains of any one biovar (Table 2). Some biovar 5 strains were not inducible and predominantly produced BlaA regardless of induction. After induction, inducible strains of biovars 2 and 5 expressed mainly BlaB (Table 2). There was no evidence for other β -lactamases besides BlaA and BlaB in all the strains examined (the total inhibition with clavulanic acid and aztreonam for each strain was 100%).

Discussion

Y. enterocolitica strains belonging to biovars 2, 4 and 5 all possessed the genes for both BlaA and BlaB β -lactamases. It is worth mentioning that most biovar 5 strains were not from clinical sources. *blaA* and *blaB* genes were even detectable in the few ampicillin-sensitive *Yersinia* strains (phenotypic group 5-IV). These results are in agreement with a previous study by De Prieta *et al.*, who found chromosomal sequences homologous to both β -lactamase genes in 12 isolates belonging to different *Y. enterocolitica* serogroups [22]. Nevertheless, the present study demonstrated that expression of the enzymes varied with the biovar and, within biovars 2 and 5, with the individual strain.

Uniform profiles of β -lactamase expression, indicative of expression of both enzymes, were found among the biovar 4 strains. This agrees with previous studies on European biovar 4 isolates [11, 23, 24], whereas Pham *et al.* found only BlaA in Australian strains [14, 17]. Further studies confirmed that European, Asian, Brazilian and South African biovar 4 isolates contained β -lactamases A and B, whereas isolates from Australia and New Zealand contained only β -lactamase A [25]. This difference was reflected in antibiogram data: MICs of amoxycillin-clavulanate and cefoxitin were 8–32 mg/L and 4–32 mg/L, respectively, for European

biovar 4 isolates, whereas Australian strains were highly susceptible to amoxycillin-clavulanate (MIC 0.5 mg/L) and cefoxitin (1–2 mg/L) [25]. Susceptibility to amoxycillin-clavulanate can be explained by the absence of enzyme B which, as an AmpC type, is resistant to inhibition by clavulanate. A previous study found that German biovar 4 strains phenotypically resembled the Australian rather than European isolates in being susceptible to amoxycillin-clavulanate [10]. The present study explains this finding; in uninduced biovar 4 strains, 75% of the β -lactamase activity was inhibited by clavulanate and so was attributable to BlaA and only the remainder was attributable to BlaB (Table 2). Although the increase of activity after induction was low, all the biovar 4 strains were inducible. Assuming that the *blaA* and *blaB* genes occur in all *Y. enterocolitica* biovar 4 isolates worldwide, it remains to be elucidated why β -lactamase expression (and MIC values) vary with the geographical origin.

The present study found a correlation between MIC data and the β -lactamase induction and inhibition tests, but in most cases it was not possible to predict the β -lactamase(s) expressed from the MIC data. Without induction, ticarcillin-resistant strains of biovar 2 belonging to the same phenotypic group (2-I) variously produced only BlaB (cluster B), predominantly BlaB (cluster A₁) or predominantly BlaA (cluster A₂ and A₃) (Table 2). Ticarcillin resistance among cluster B strains within the phenotypic group 2-I is surprising, because several studies have shown that sensitivity to ticarcillin is linked to the sole production of BlaB [14, 17]. It remains to be examined whether other mechanisms, e.g., impermeability, contribute to β -lactam resistance in these strains. In this study and others [14, 17] ticarcillin-sensitive strains (phenotypic group 2-II and 5-IV) produced only BlaB; however, ticarcillin-sensitive *Y. enterocolitica* strains that produce both BlaA and BlaB are seen (unpublished data). It remains to be determined why clavulanate was an effective inhibitor for ticarcillin-resistant biovar 4 and phenotypic groups 5-I and 5-II strains producing predominantly BlaA, whereas it was not effective for phenotypic group 2-I, cluster A₂ and A₃ also producing predominantly BlaA. This discrepancy may reflect additional mechanisms contributing to ticarcillin resistance or to the fact that conditions in induction and inhibitory tests were not equivalent to those in MIC determinations.

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