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Rapid Identification of Metallo- and Serine β-Lactamases

DAVID J. PAYNE,* REBECCA CRAMP, JOHN H. BATESON, JANE NEALE, AND DAVID KNOWLES

SmithKline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey RH3 7AJ, United Kingdom

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Simple methods to detect, identify, and differentiate metallo- and serine β-lactamases were developed and used to differentiate enzymes produced by 17 clinical isolates of *Xanthomonas maltophilia*. All isolates exhibited β-lactamase activity, and in 16 strains this was induced by imipenem. All but one isolate hydrolyzed imipenem (and meropenem), and in all cases this activity was inhibited by 1 mM EDTA. The metallo- and serine β-lactamases in the cell extracts were distinguished on isoelectric focusing (IEF) gels by using the following procedures. (i) Cell lysates were preincubated with 83 mM EDTA prior to IEF and subsequent visualization with nitrocefin, and (ii) after IEF, the gels were overlaid with either 1 mM zinc sulfate or 100 μM BRL 42715 before staining with nitrocefin. Bands of β-lactamase activity which were removed by BRL 42715 but unaffected by EDTA or zinc sulfate were categorized as serine β-lactamases. Bands which were unaffected by BRL 42715 but inhibited by EDTA or enhanced by zinc sulfate were classified as metallo-β-lactamases. By using this approach, seven metallo-β-lactamases were differentiated with pI values of 4.8 (two strains), 5.5 (four strains), 5.7 (one strain), 6.0 (one strain), 6.4 (four strains), 6.6 (one strain), and 6.8 (three strains). The metallo-β-lactamase band with a pI of 6.4 aligned with the recently characterized metallo-β-lactamase from *X. maltophilia* 511. Heterogeneity was also observed for the serine β-lactamases: 14 isolates elaborated serine β-lactamase activity which focused with major bands with at least eight different pIs. The remaining three strains produced serine β-lactamases which focused with five distinct bands with pIs of 6.4, 6.2, 5.7, 5.5, and 5.2. We conclude that *X. maltophilia* produces many types of metallo- and serine β-lactamases distinguishable by these new methods and that the previously reported L-1 and L-2 enzymes are not solely representative of the β-lactamases produced by this species.

Xanthomonas maltophilia is becoming recognized as an important nosocomial pathogen, and most clinical isolates are resistant to almost all antibiotics (10 and references therein). In addition, Elting et al. (5) demonstrated that patients given imipenem had a 10-fold greater chance of becoming infected with *X. maltophilia* than did a control group. For these reasons, the treatment of *X. maltophilia* infections is considered to have reached a crisis point (9).

X. maltophilia GN12873 was shown to produce a cephalosporinase, L-2 (13), and a metallo-β-lactamase, L-1 (14). The L-1 enzyme, which hydrolyzes most classes of β-lactam antibiotics, is thought to be largely responsible for the resistance to carbapenems exhibited by this species. Indeed, an L-1-deficient strain of *X. maltophilia* is 250 times more susceptible to meropenem, whose MIC is 0.5 μg/ml for that strain (1).

There are conflicting data on the occurrence of metallo-β-lactamases in *X. maltophilia*. Initially, it was thought that all strains of *X. maltophilia* produced the L-1 enzyme which was characterized by Saino et al. (14) and N terminally sequenced by Bicknell et al. (2). However, Cullmann and Dick (4) examined 20 clinical isolates and identified six different β-lactamases by their differing pIs. All of the strains studied produced enzymes which hydrolyzed imipenem and meropenem. However, inhibition of carbapenem hydrolysis by 1 mM EDTA could not be demonstrated for any of the 20 enzymes and since 0.1 mM EDTA caused 93% inhibition of the L-1 metallo-β-lactamase (14), it was suggested that the carbapenemase resistance of *X. maltophilia* may not be conferred by a metallo-β-lactamase. The purpose of this work was to develop a simple method to investigate the nature of β-lactamases produced by clinical isolates of *X. maltophilia*.

* Corresponding author. Mailing address: SmithKline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey RH3 7AJ, United Kingdom. Phone: 737-364509. Fax: 737-364597.

MATERIALS AND METHODS

Bacterial strains. All of the clinical isolates of *X. maltophilia* used in this study were identified by API and were obtained from hospitals within the United Kingdom. *X. maltophilia* 511 is a control strain and produces the metallo-β-lactamase characterized by Felici et al. (6). *Escherichia coli* K-12 strain J53-2 produced TEM-1.

Antibacterial compounds. Ceftazidime was a gift from Glaxo, ceftriaxone was from Roche, ciprofloxacin was from Bayer, imipenem was from MSD, meropenem was from ICI, and cefpirome was from Hoechst. Nitrocefin and BRL 42715 were prepared in our own laboratories.

Antibacterial susceptibilities. MIC determinations were performed by the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (8).

Growth of cultures and preparation of β-lactamases. Ten-milliliter aliquots of Nutrient Broth No. 2 (Oxoid, Basingstoke, United Kingdom) was inoculated with each of the *X. maltophilia* strains and the *E. coli* TEM-1 producer. These cultures were incubated overnight at 30°C in an orbital shaker. One milliliter of each overnight culture was then added to 2 × 100 ml of Nutrient Broth No. 2 and grown at 30°C for 2 h. Imipenem (final concentration, 5 μg/ml) was then added to one of each pair of cultures. Following a further 2.5 h of growth, the cells were harvested and washed with 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 7.0. The final pellet was resuspended in 1 ml of 25 mM PIPES buffer, pH 7.0, and subjected to ultrasonication. The resultant lysate was cleared by centrifugation to provide the β-lactamase preparation (12).

Determination of specific activity. The protein concentra-

TABLE 1. Antibacterial susceptibilities of *X. maltophilia* clinical isolates

X. maltophilia strain	MIC (μg/ml) of:					
	Ceftazidime	Cefpirome	Ceftriaxone	Meropenem	Imipenem	Ciprofloxacin
511	>64	>64	>64	>64	>64	>0.5
GEL	16	>64	>64	>64	>64	1
B51384	2	8	32	>64	>64	4
LL284	2	64	64	>64	>64	2
GE5	4	64	64	>64	>64	1
0062	64	>64	>64	>64	>64	16
00107	64	>64	>64	>64	>64	16
U152	64	>64	>64	>64	>64	0.5
J2323	>64	64	64	>64	>64	4
00157	>64	>64	>64	>64	>64	8
37	>64	>64	>64	>64	>64	1
10257	64	>64	>64	>64	>64	0.5
10258	4	4	64	>64	64	1
ED136	>64	>64	>64	64	>64	0.5
H-25	>64	>64	>64	>64	>64	1
A37454	>64	>64	>64	>64	>64	1
00141	>64	>64	>64	>64	>64	1
AA158	8	64	>64	1	16	8

tion of each cell extract was determined by the Bio-Rad Protein Estimation Kit. The β-lactamase activity of each preparation was determined by measuring the initial rate of hydrolysis of 100 μM nitrocefin. All enzyme assays were performed in 25 mM PIPES buffer, pH 7.0. The β-lactamase preparations from induced cultures had to be diluted between 10 and 1,000 times in 25 mM PIPES buffer, pH 7.0, to achieve an optimal rate of nitrocefin hydrolysis. With the exception of the extract from *X. maltophilia* GEL, the rates of hydrolysis of nitrocefin by the preparations from noninduced cultures were measured with undiluted samples. Specific activity was expressed as the rate of hydrolysis of nitrocefin per milligram of protein (nanomoles per minute per milligram of protein).

Biochemical characterization of β-lactamase preparations. The initial rates of hydrolysis of β-lactams (100 μM) by the each of the cell extracts were measured with a Beckman DU7400 spectrophotometer. The rates of meropenem and imipenem degradation were measured at 300 and 299 nm, respectively. To achieve optimum rates of hydrolysis, the extracts were diluted as described above. The initial rate of hydrolysis of imipenem was also measured following 10 min of preincubation (37°C) of the enzyme preparation with 1 mM (final concentration after addition of the substrate) EDTA. The level of inhibition caused by the EDTA, which is a known metal chelator, was expressed as a percentage of the rate of imipenem hydrolysis by the untreated control. The 50% inhibitory concentrations (I_{50}s) of BRL 42715, EDTA, and zinc sulfate for TEM-1 and the β-lactamases produced by *X. maltophilia* 511 were measured following enzyme and inhibitor preincubation as described by Payne et al. (11).

Characterization of metallo-β-lactamases by isoelectric focusing (IEF). β-Lactamase preparations from both induced and noninduced cultures were examined by IEF as described by Matthew et al. (7). Filter paper applicator tabs were used to load all of the samples onto IEF gels. The isoelectric points of the *X. maltophilia* enzymes were measured from a plot of distance migrated versus pI for known proteins by using a pH 4.7 to 10.6 pI calibration kit (BDH Chemicals Ltd., Poole, England). Three methods were used to differentiate the *X. maltophilia* β-lactamases on IEF gels. (i) To investigate the effects of EDTA, a 10-μl aliquot of the crude lysate was mixed with 2 μl of 0.5 M EDTA in 25 mM PIPES buffer, pH 7.0 (final EDTA concentration, 83 mM), and another 10-μl aliquot was mixed with 2 μl of 25 mM PIPES buffer, pH 7.0. Both of these samples were then loaded and focused on the IEF gel and visualized in the normal manner with nitrocefin (0.5 mg/ml). (ii) The IEF gels were overlaid with filter paper soaked in 25 mM PIPES buffer, pH 7.0, with 1 mM zinc sulfate before staining with nitrocefin to enhance the identification of any zinc-dependent β-lactamases. (iii) Prior to nitrocefin staining, gels were overlaid with filter paper soaked in 25 mM PIPES buffer, pH 7.0, with 100 μM BRL 42715, which is a potent inhibitor of serine β-lactamases (3). Enzyme preparations from the *X. maltophilia* 511 and *E. coli* K-12 strain J53-2 TEM-1 were also subjected to each of these protocols as controls.

RESULTS

Antibacterial susceptibilities and β-lactamase induction. The MICs of imipenem and meropenem for *X. maltophilia* AA158 were 16 and 1 μg/ml, respectively, and the MICs of carbapenems for all of the other strains were greater than or equal to 64 μg/ml (Table 1). *X. maltophilia* GEL was the only clinical isolate which produced high levels of β-lactamase constitutively, and induction caused only a fivefold increase in β-lactamase activity in strain U152. All of the other strains showed a >40-fold increase in β-lactamase activity following induction with imipenem (Table 2). The control strain, *X. maltophilia* 511, produced high levels of β-lactamase constitutively, and imipenem caused no induction of TEM-1. The β-lactamase activity in most of the extracts from noninduced cultures was very low. Therefore, substrate profile determinations were performed on extracts from the induced cultures.

Biochemical characterization of β-lactamase preparations. Of the 17 extracts from the clinical isolates, 16 hydrolyzed both meropenem and imipenem. Extracts from seven of the isolates and the extract from *X. maltophilia* 511 hydrolyzed meropenem at a rate greater than or equal to that for imipenem. The hydrolysis of imipenem by these 16 preparations and the extract from *X. maltophilia* 511 was inhibited between 57 and 99% by 1 mM EDTA relative to the untreated control (Table 2). No detectable hydrolysis of carbapenems by the extract from *X. maltophilia* AA158 and *E. coli* J53-2 (TEM-1) was observed.

TABLE 2. Biochemical assessment of β-lactamase extracts from clinical isolates of *X. maltophilia*, *X. maltophilia* 511, and TEM-1-producing *E. coli*

Strain	Sp act (nmol/min/mg)		Induction ratio (induced/noninduced sp act)	Initial rate of hydrolysis of imipenem (nmol/min/mg)	Hydrolysis of meropenem relative to imipenem (%)	Inhibition of imipenem hydrolysis by 1 mM EDTA (%)
	Noninduced	Induced				
E. coli (TEM-1 producing)	320			<0.1		
X. maltophilia 511	5,717	6,457	1	1,347	204	92
X. maltophilia GEL	2,783	2,670	0	1,783	73	91
X. maltophilia B51384	3.9	173	44	374	229	64
X. maltophilia LL284	4.8	2,766	576	56	59	89
X. maltophilia GE5	6.2	4,651	750	69	49	99
X. maltophilia 0062	4.7	1,345	286	80	109	87
X. maltophilia 00107	7.8	1,318	169	5,441	61	71
X. maltophilia U152	525	2,562	5	1,639	62	90
X. maltophilia J2323	15	994	66	5,550	59	96
X. maltophilia 00157	3.9	1,120	287	86	99	73
X. maltophilia 37	6.0	631	105	90	104	92
X. maltophilia 10257	8.3	5,084	613	8,954	24	64
X. maltophilia 10258	6.6	296	45	236	129	61
X. maltophilia ED136	15	1,864	124	55	244	67
X. maltophilia H-25	3.3	3,467	1,051	333	97	78
X. maltophilia A37454	13	2,643	203	12,240	28	57
X. maltophilia 00141	9.5	2,816	296	1,962	58	62
X. maltophilia AA158	22	2,855	130	<0.1		

TEM-1 and the serine β-lactamase produced by *X. maltophilia* 511 were sensitive to inhibition by BRL 42715 (I_{50}, <0.005 μM), but both enzymes were resistant to inhibition by EDTA (I_{50}, >167 mM), and neither enzyme was activated by 1 mM zinc sulfate. The metallo-β-lactamase from strain 511 (pI 6.4) was not inhibited by BRL 42715 (I_{50}, >100 μM) but was sensitive to EDTA (I_{50}, 5 μM). This enzyme was activated threefold by 1 mM zinc sulfate. Consequently, these results illustrate that EDTA, zinc sulfate, and BRL 42715 can be used to differentiate between metallo- and serine β-lactamases and illustrate the rationale behind the IEF assays described below.

IEF of cell extracts. IEF illustrated that all of the cultures of *X. maltophilia* produced the same β-lactamase bands irrespective of whether they were induced or noninduced. With the exception of those of strain GEL, all of the bands seen in extracts from noninduced cultures were weaker than the corresponding bands in the extracts of the induced cultures. Therefore, all further IEF analyses were performed on extracts from induced cultures.

Repeated attempts to focus the β-lactamases from the *X. maltophilia* strains failed to achieve narrowly focused bands like those commonly obtained with TEM-1. It is possible that these broader bands represent more than one enzyme or are artifacts of the extraction and IEF procedures. Further investigations are required to elucidate the nature of these bands. Consequently, for this work, each pI quoted refers to the most intense part of the β-lactamase band. IEF of the *X. maltophilia* extracts showed that this species produced β-lactamases with a wide variety of pI values (Table 3). IEF assays using the conventional protocol (Table 3, column 2) illustrated that 9 of the 17 clinical isolates focused with two main bands, one with a pI greater than or equal to 7.7 and the second with a pI less than or equal to 6.6 (Table 3, column 2). Only one β-lactamase band with a high pI was visualized in *X. maltophilia* LL284, GE5, 0062, and 00107, and the extract from strain AA158 focused as a series of bands from pI 8.5 to pI 5.5 (Table 3, column 2). Extracts from *X. maltophilia* H25, 00141, and A37454 had identical IEF patterns, with six bands between pI 6.8 and pI 5.2 (Table 3, column 2). A similar IEF pattern for the β-lactamases produced by *X. maltophilia* H25 has been observed previously (9a).

Effects of EDTA on visualization of β-lactamase activity by IEF. Examination of the enzyme preparations from *X. maltophilia* 511 on IEF with and without EDTA illustrated that the metallo-β-lactamase activity with a pI of 6.4 was absent in the EDTA-treated sample and the band with a pI of 9.7 remained unaffected (Fig. 1). The EDTA treatment had no effect on the TEM-1 β-lactamase. Figure 1 shows extracts from seven of the clinical isolates focused with and without EDTA, and in each case one of the bands of β-lactamase activity was removed by the EDTA treatment, leaving the other band(s) unaffected. Only the top band (pI 6.8) was eliminated from the IEF patterns of *X. maltophilia* H25, and the extracts from *X. maltophilia* A37454 and 00141 behaved similarly (Table 3). Overall, the EDTA treatment prevented visualization of a band in 12 of the 17 clinical isolates (Table 3). A total of six different EDTA-sensitive enzymes were observed, and these had pIs of 4.8 (two strains), 5.7 (one strain), 6.0 (one strain), 6.4 (four strains), 6.6 (one strain), and 6.8 (three strains) (Table 3). The β-lactamases with a pI of 6.4 were focused side by side with the metallo-β-lactamase from *X. maltophilia* 511, and in all cases the enzymes aligned with the strain 511 β-lactamase (data not shown).

Effects of zinc sulfate overlay on visualization of β-lactamase activity by IEF. EDTA had no effect on the β-lactamase bands visualized in extracts from *X. maltophilia* LL284, GE5, 0062, and 00107 (Table 3). However, when the gel was overlaid with 1 mM zinc sulfate prior to staining with nitrocefin, a zinc-dependent β-lactamase with a pI of ca. 5.5 was visualized in the extract from each of these four strains (Table 3). Exposure of the other focused extracts to a 1 mM zinc sulfate overlay caused the bands already visualized to become more intense.

Effect of BRL 42715 overlay on visualization of β-lactamase activity. The IEF gel in Fig. 2a was overlaid with 1 mM zinc sulfate prior to staining and shows the β-lactamase activity of the extract from strain 511 along with the extracts which elaborated the seven different types of enzymes which the previous experiments have illustrated to be either inactivated by EDTA or activated by zinc sulfate. Another gel loaded with the same extracts was overlaid with 100 μM BRL 42715 in addition to zinc sulfate prior to staining with nitrocefin (Fig.

TABLE 3. Effects of EDTA, zinc sulfate, and BRL 42715 treatments on β-lactamases from 17 clinical isolates of *X. maltophilia*, *X. maltophilia* 511, and TEM-1-producing *E. coli*

Strain	pI(s) of band(s) seen on conventional IEF	Band seen when prep focused with EDTA	Band(s) seen after treatment with zinc sulfate	Bands seen after treatment with BRL 42715	pI of metallo-β-lactamase (type)
E. coli K-12 J53-2 (TEM-1)	5.4	5.4	5.4		
X. maltophilia 511 (L-1)	6.4, 9.7	9.7	6.4, 9.7	6.4	6.4
X. maltophilia GEL	4.8, 10.3	10.3	4.8, 10.3	4.8	4.8 (1)
X. maltophilia B51384	4.8, 9.7	9.7	4.8, 9.7	4.8	4.8 (1)
X. maltophilia LL284	9.9	9.9	5.5, 9.9	5.5	5.5 (2)
X. maltophilia GE5	9.9	9.9	5.5, 9.9	5.5	5.5 (2)
X. maltophilia 0062	8.6	8.6	5.5, 8.6	5.5	5.5 (2)
X. maltophilia 00107	8.9	8.9	5.5, 8.9	5.5	5.5 (2)
X. maltophilia U152	5.7, 8.6	8.6	5.7, 8.6	5.7	5.7 (3)
X. maltophilia J2323	6.0, 7.7	7.7	6.0, 7.7	6.0	6.0 (4)
X. maltophilia 00157	6.4, 9.6	9.6	6.4, 9.6	6.4	6.4 (5)
X. maltophilia 37	6.4, 9.6	9.6	6.4, 9.6	6.4	6.4 (5)
X. maltophilia 10257	6.4, 9.9	9.9	6.4, 9.9	6.4	6.4 (5)
X. maltophilia 10258	6.4, 9.7	9.7	6.4, 9.7	6.4	6.4 (5)
X. maltophilia 136	6.6, 10.0	10.0	6.6, 10.0	6.6	6.6 (6)
X. maltophilia H-25	6.8[a]	*a*	6.8[a]	6.8	6.8 (7)
X. maltophilia A37454	6.8[a]	*a*	6.8[a]	6.8	6.8 (7)
X. maltophilia 00141	6.8[a]	*a*	6.8[a]	6.8	6.8 (7)
X. maltophilia AA158	5.5–8.5	No changes	5.5–8.5	None	

[a] In each case, the same banding pattern was observed; bands with pIs of 6.4, 6.2, 5.7, 5.5, and 5.2 were identified.

2b). This illustrated that BRL 42715 had no effect on any of the bands which were either EDTA sensitive or activated by zinc sulfate but eliminated the activity of those bands unaffected by these two treatments (Table 3).

All 17 clinical isolates produced BRL 42715-sensitive enzymes which were unaffected by EDTA or zinc sulfate. Four focused at pI 9.7 or 9.6, four focused at pI 9.9 or 10.0, two focused at pI 8.6, and four others focused at another four different pIs (Table 3). The remaining three isolates produced identical multiple bands which were sensitive to BRL 42715.

DISCUSSION

β-Lactamase activity was markedly increased following induction with imipenem in all of the strains investigated, except *X. maltophilia* GEL, which produced high levels of the enzyme without induction. This suggests that most multiply resistant clinical isolates of *X. maltophilia* possess inducible β-lactamases and that *X. maltophilia* GEL may have a mutation in its regulatory gene(s) which causes high enzyme levels to be produced constitutively. IEF of β-lactamase preparations from induced and uninduced cultures showed that in all cases induction was a result of an increase in production of all of the β-lactamase bands seen on the gel and was not exclusively associated with one of the β-lactamases produced by the strain.

Extracts from induced cultures of 16 strains hydrolyzed the carbapenems, meropenem, and imipenem. Hydrolysis of imipenem was inhibited by 1 mM EDTA, providing strong evidence for the involvement of metallo-β-lactamases. Cullmann and Dick (4) failed to detect EDTA inhibition of the imipenemase activity of β-lactamases from 20 clinical isolates of *X. maltophilia*. This may be explained if their β-lactamase preparations contained high levels of metal ions and other

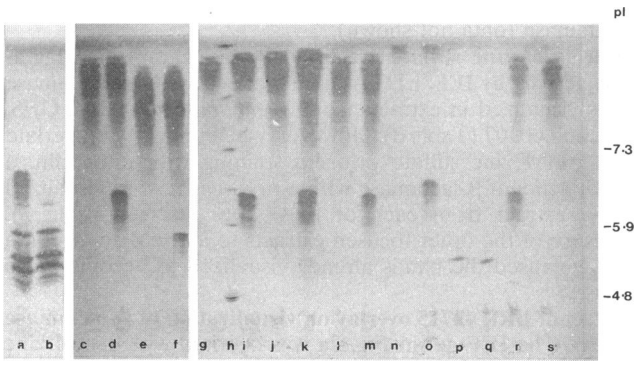

FIG. 1. Effect of 83 mM EDTA on the IEF of β-lactamases from *X. maltophilia*. Lanes: a, *X. maltophilia* A37454; b, A37454 plus EDTA; c, 37 plus EDTA; d, 37; e, 152 plus EDTA; f, 152; g, 10258 plus EDTA; h, pI markers; i, 10258; j, 10257 plus EDTA; k, 10257; l, 00157 plus EDTA; m, 00157; n, ED136 plus EDTA; o, ED136; p, TEM-1 plus EDTA; q, TEM-1; r, 511 (known metallo-β-lactamase producer); s, 511 plus EDTA.

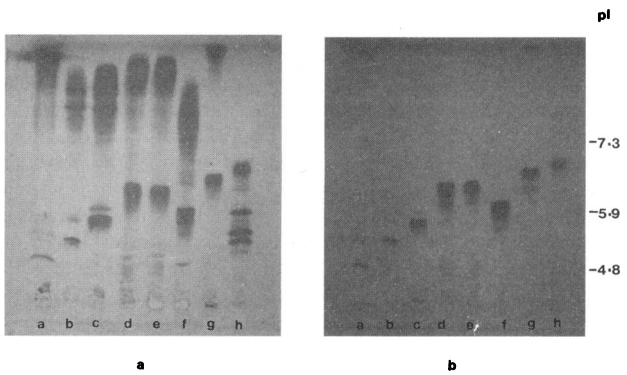

FIG. 2. IEF of *X. maltophilia* extracts showing each of the seven different types of metallo-β-lactamases identified in this survey. Lanes: a, *X. maltophilia* GEL; b, 0062; c, U152; d, 511 (known metallo-β-lactamase producer); e, 37; f, J2323; g, 136; h, A37454. Panels: a, gel overlaid with 1 mM zinc sulfate and stained with nitrocefin; b, gel overlaid with 1 mM zinc sulfate and BRL 42715 prior to nitrocefin staining.

material which would interact with the EDTA. However, our measurements were performed with a higher concentration of EDTA and with extracts that were diluted 10 to 1,000 times so the amount of cell extract in the assay was minimized.

Previously, it has been shown that overlaying IEF gels with specific inhibitors can distinguish different types of serine β-lactamases (15). We developed similar procedures to identify the metallo- and serine β-lactamases produced by 17 clinical isolates of *X. maltophilia*. Bands not visualized following pretreatment with 83 mM EDTA were considered to be metallo-β-lactamases. IEF gels overlaid with 1 mM zinc sulfate revealed other metallo-β-lactamases not seen by conventional IEF. Either these enzymes require more Zn^{2+} to remain active, or their Zn^{2+} ions are bound less tightly. As both the method used to prepare the β-lactamase samples and the IEF of the samples reduced the level of available Zn^{2+}, treatment of the gels with zinc sulfate provides sufficient Zn^{2+} to activate the enzyme so that it can hydrolyze nitrocefin. Overlaying of IEF gels with zinc sulfate has not been adopted in any other surveys of the β-lactamases of *X. maltophilia*, so these types of enzymes may have been overlooked in other studies. The only β-lactamases not effectively inhibited by BRL 42715 are metallo-β-lactamases; in fact BRL 42715 is a substrate for metallo-β-lactamases (6a). On the basis of these observations, bands inhibited by BRL 42715 were proposed to be serine β-lactamases and those unaffected by this treatment were proposed to be metallo-β-lactamases.

On the basis of our studies, we recommend that only two assays need to be performed for rapid identification of metallo- and serine β-lactamases on IEF. (i) The extract must be focused with and without 100 mM EDTA, and (ii) the focused extract must be overlaid with a mixture of 1 mM zinc sulfate and 100 μM BRL 42715 prior to staining with nitrocefin.

None of the β-lactamase bands produced by *X. maltophilia* AA158 were affected by EDTA or zinc sulfate, but the band was sensitive to inhibition by BRL 42715. The cell extract had no carbapenemase activity, and the MICs of meropenem and imipenem for the strain were similar to those for a mutant of *X. maltophilia* which was metallo-β-lactamase deficient (1). Therefore, the AA158 strain probably does not produce a metallo-β-lactamase.

The IEF methods showed that the 16 other clinical isolates produced seven different metal-dependent β-lactamases which have been provisionally designated enzyme types 1 to 7 (Table 3). The four strains which produced the metallo-β-lactamase with a pI of 5.5 came from the same source. However, strains producing enzymes with pIs of 4.8, 6.4, and 6.8 were obtained from more than one origin. This shows that the different enzyme types were not localized to one area but were present in different clinical populations of *X. maltophilia*. Heterogeneity of the serine β-lactamases was also observed. The only previously published serine enzyme in *X. maltophilia* was L-2, with a pI of 8.4 (13). However, even allowing for irregularities in the IEF gels, 14 of the clinical isolates produced at least seven different β-lactamases with pIs ranging from 7.7 to 10.3. These were unaffected by EDTA or zinc sulfate but eliminated by BRL 42715. In addition, the five β-lactamase bands (pIs 5.5 to 6.4) produced by *X. maltophilia* H-25, 00141, and A37254 all exhibited the same characteristics of serine-active site enzymes (unaffected by zinc sulfate or EDTA but eliminated by BRL 42715). It is not possible to deduce from IEF gels whether these five bands represent different β-lactamases or satellite bands of the same β-lactamase.

Although the various metallo- and serine β-lactamases identified in this survey have been differentiated by pI, it cannot be assumed that the seven different types of enzymes have diverse substrate and inhibition profiles. It is possible that, as with TEM and SHV β-lactamases, the diversity of pIs arose from only a few amino acid changes. Therefore, further work is required to ascertain the relationships among these different metallo-β-lactamases.

Other workers have reported heterogeneity in the pI values of the β-lactamases produced by *X. maltophilia*, but the developments described here readily distinguish between metallo and serine active-site enzymes. The heterogeneity observed shows that many isolates of *X. maltophilia* produce metallo- and serine β-lactamases with pIs different from those of the L-1 and L-2 enzymes originally described by Saino et al. (13, 14). These novel detection methods will also facilitate larger surveys of *X. maltophilia* strains to deduce the most prevalent metallo-β-lactamase in this species. It is possible that these procedures are also applicable for the identification of metallo- and serine β-lactamases in other species. However, BRL 42715, zinc sulfate, and EDTA may have different effects on the β-lactamases produced by other species of bacteria, and appropriate method development studies would have to be conducted.

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