ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2010, p. 2219–2224 Vol. 54, No. 5
0066-4804/10/$12.00 doi:10.1128/AAC.00816-09

Efflux Pumps, OprD Porin, AmpC β-Lactamase, and Multiresistance in *Pseudomonas aeruginosa* Isolates from Cystic Fibrosis Patients▽

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Received 18 June 2009/Returned for modification 20 September 2009/Accepted 20 February 2010

Expression of *ampC*, *oprD*, *mexA*, *mexC*, *mexE*, and *mexX* was studied in 25 *Pseudomonas aeruginosa* isolates from cystic fibrosis patients, including 14 isolates of the Liverpool epidemic strain. Overexpressed *mexA* or *ampC* and reduced *oprD* were associated with β-lactam resistance. A specific combination of *mexR*, *nalC*, and *nalD* mutations occurred in 11 Liverpool strain isolates, including 7 with upregulated *mexA*.

Once established in the respiratory tree of the cystic fibrosis (CF) patient, *Pseudomonas aeruginosa* survives aggressive chemotherapy, which may select for resistance-conferring mutations (28). Several mutations compromise β-lactams, including those that (i) upregulate AmpC β-lactamase (13, 24), (ii) inactivate or downregulate the carbapenem-specific OprD porin (13, 14, 22), or (iii) upregulate efflux by MexAB-OprM (8, 11, 22) and other resistance-nodulation-cell division (RND) pumps (1, 19–21, 30). Most of these pumps also excrete quinolones and one, MexXY-OprM, acts against aminoglycosides. While one can usually infer the resistance mechanisms of non-CF *P. aeruginosa* isolates by interpretive reading of antibiograms (17), this fails for CF isolates, which often have pan-resistance or antibiogram profiles discordant with known mechanisms.

We investigated the combinations of resistance mechanisms in 25 CF patient *P. aeruginosa* isolates, including 14 representatives of the Liverpool epidemic strain (2) (Fig. 1), which is disseminated among United Kingdom CF patients. Selection was based on high or low meropenem MICs among isolates referred to the Health Protection Agency's Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL) during 2007 and 2008. Susceptibility was determined using the British Society for Antimicrobial Chemotherapy agar dilution methodology or Etest and categorized using (i) European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints, with a biological breakpoint of 64 μg/ml for cefotaxime to distinguish normal insensitivity (MICs of 8 to 32 μg/ml) from substantial resistance or (ii) a biological cutoff MIC >4× the mode for wild-type *P. aeruginosa* (http://www.srga.org/eucastwt/MICTAB/). We used *P. aeruginosa* PAO1 as a control along with seven strains with varied expression levels of resistance determinants: strains M1251 and R70 had upregulated efflux (15, 16); R20 (29) had normal efflux activity; AHP and Z799/61 (31) had reduced efflux; strains 2779-con and 1405-con D2$^-$ overexpressed AmpC β-lactamase (13), and the latter also lacked OprD porin.

Amplification of β-lactamase genes used primers for bla_{TEM} (T-1, 5′-ATG AGT ATT CAA CAT TTC CG-3′; T-2, 5′-CCA ATG CTT AAT CAG TGA CG-3′), bla_{SHV} (S-1, 5′-TCA GCG AAA AAC ACC TTG-3′; S-2, 5′-TCC CGC AGA TAA ATC ACC A-3′), or those described previously for IMP, VIM, SPM-1, GIM-1, and SIM-1 metallo-β-lactamase genes (5). Carbapenemase phenotypes were sought by (i) agar dilution imipenem-EDTA (320 μg/ml) synergy tests and (ii) a modified Hodge test (3, 10) with imipenem and meropenem disks (10 μg) on Mueller-Hinton (MH) agar inoculated with *Escherichia coli* ATCC 25922.

Real-time reverse transcription-PCR (RT-PCR), with the primers detailed in Table 1, was used to quantify transcription of genes encoding efflux pump proteins MexA, MexC, MexE, and MexX, outer membrane protein OprD, and AmpC β-lactamase. Total RNA was isolated from late-log-phase cultures with RNeasy kits (Qiagen Inc., Crawley, United Kingdom) and treated with DNase (1 U/μl). RT-PCR was performed in triplicate using different RNA extractions (50 ng RNA per reaction mixture), LightCycler FastStrand DNA Master SYBR green I (Roche, Mannheim, Germany), and HotStarTaq Plus DNA polymerase (Qiagen). Gene expression was normalized versus that of *rpoD* in the same strain and then calibrated relative to *P. aeruginosa* PAO1 (27), which was assigned a value of 1.0. Increases or decreases in gene expression of ≥2-fold and ≤0.5-fold were taken as significant, and Student's *t* test was used to compare the occurrence of these changes between susceptible and resistant groups. Isolates with intermediate susceptibility were regarded as susceptible. Variables associated significantly ($P \leq 0.05$) with resistance in this univariate analysis were used as covariates in multivariate logistic regression (SPSS v.16.0; Analytical Software, St. Paul, MN).

The *mexR*, *nalC*, and *nalD* genes were amplified using the primers listed in Table 1 and previously described conditions (23, 24, 26); sequences were determined with a CEQ 8000 XL genetic analysis system (Beckman Coulter, High Wycombe, United Kingdom) and compared with those of *P. aeruginosa* PAO1 (www.ncbi.nlm.gov/BLAST/).

Antibiotic susceptibilities and gene expression data are shown in Table 2. Control strains M1251 and R70, with upregulated efflux (11), had corresponding phenotypes, although

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▽ Published ahead of print on 1 March 2010.

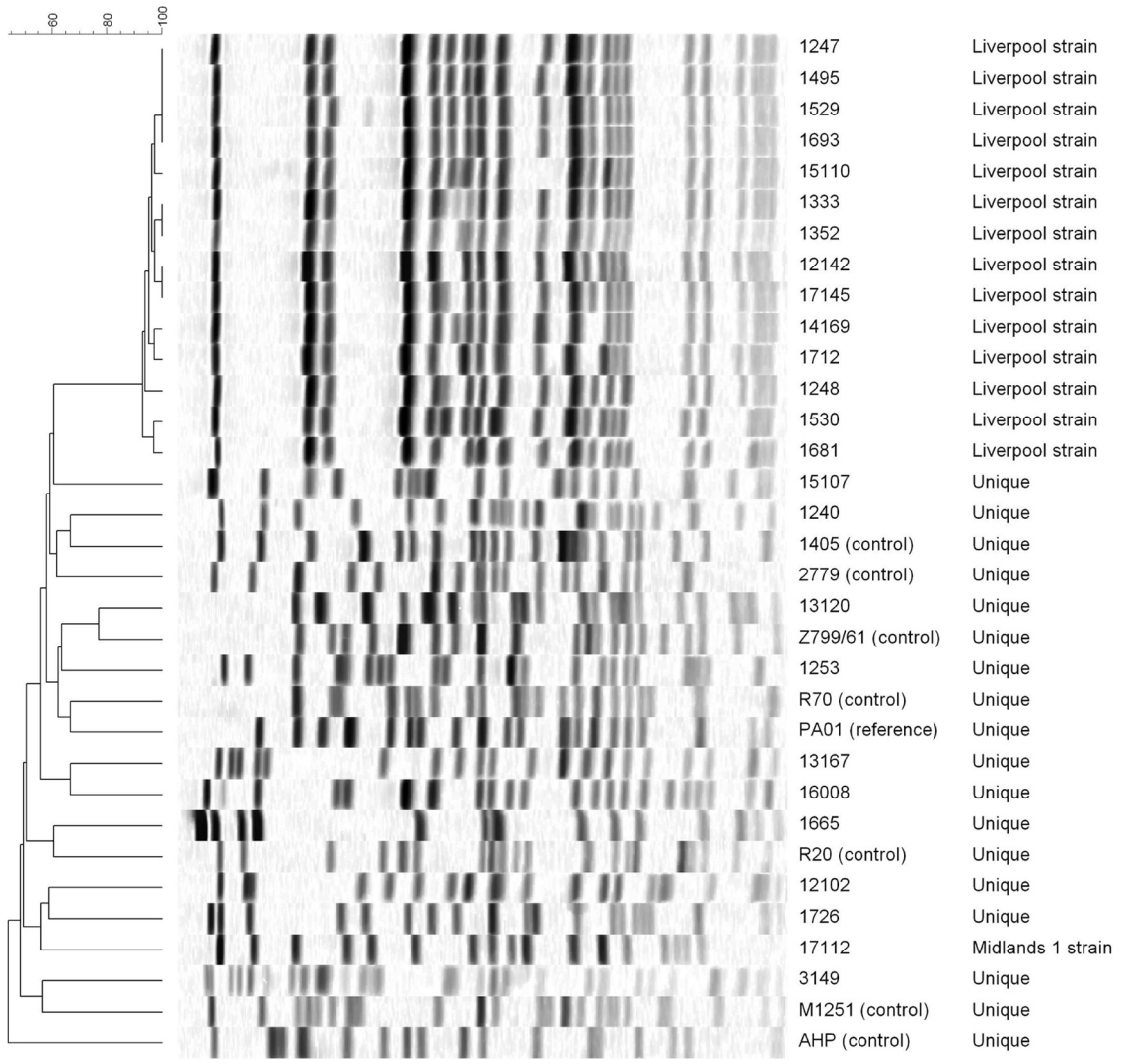

FIG. 1. Pulsed-field gel electrophoresis banding patterns of SpeI-digested genomic DNA from control strains and clinical isolates of *P. aeruginosa*.

R70 was additionally resistant to imipenem. The present analysis found overexpression of *mexA* (7.28-fold and 5.94-fold, respectively) and *mexX* (2.70- and 7.94-fold) in these strains. In addition, R70 had reduced OprD expression, correlating with imipenem resistance; also, a 5- to 6-fold increase in AmpC expression was observed, which was far less than the >300-fold increase in the two derepressed controls, 2779-con and 1405-con D2$^-$ (13). Strain 1405-con D2$^-$, which showed imipenem resistance (MIC, 16 μg/ml) and, inconsistent with previous results (11), increased carbenicillin resistance (512 μg/ml), also had reduced *oprD* and increased *mexA* expression levels, explaining deviations from a straightforward AmpC phenotype. Strains AHP and Z799/61, with highly susceptible phenotypes and weak or absent efflux in direct assays (11, 12), showed weak transcription of all *mex* genes studied and of *ampC*; Z799/61 also had weak *oprD* expression but, since the organism lacks AmpC expression, this should not confer imipenem resistance (13), thus agreeing with the organism's phenotype. R20 had similar expression of most genes as PAO1, which it resembles in MIC profile, although with increased *oprD* expression.

Twelve CF isolates gave a weak positive Hodge test but with imipenem only, and two (12102 and 15110) were weakly positive in the EDTA synergy test; PCR did not detect genes encoding known metallo-β-lactamases or TEM or SHV enzymes. Although other β-lactamases, such as OXA-50 (6, 9), may have contributed to these phenotypes, we noted that 9/12 Hodge test-positive CF isolates overexpressed *ampC*. Similarly, controls R70, 2779-con, and 1405-con D2$^-$, also with increased *ampC* expression, gave weak positive Hodge tests. None of the control strains was positive in the EDTA synergy test.

The CF isolates variously had altered expression of one to six genes implicated in resistance (Table 2) and, based on univariate analysis (data not shown), their antibiograms broadly agreed with the known spectra of these mechanisms. Thus, upregulation of *mexAB-oprM* was associated with resistance to β-lactams except imipenem, whereas upregulation of

TABLE 1. PCR primers used for real-time RT-PCR and to sequence regulatory genes

Analysis method and gene	Primer	DNA sequence	Amplicon size (bp)	Reference
RT-PCR				
rpoD	For	5′-CGCAACAGCAATCTCGTCTGAAA-3′	130	This study
	Rev	5′-GCGGATGATGTCTTCCACCTGTT-3′		This study
ampC	For	5′-GGTGCAGAAGGACCAGGCACAGAT-3′	97	This study
	Rev	5′-CGATGCTCGGGTTGGAATAGAGGC-3′		This study
oprD	For	5′-CGGCGACATCAGCAACACC-3′	194	This study
	Rev	5′-GGGCCGTTGAAGTCGGAGTA-3′		This study
mexA	For	5′-GGCGACAACGCGGCGAAGG-3′	203	This study
	Rev	5′-CCTTCTGCTTGACGCCTTCCTGC-3′		This study
mexC	For	5′-GCAATAGGAAGGATCGGGGCGTTGG-3′	102	This study
	Rev	5′-CCTCCACCGGCAACACCATTTCG-3′		This study
mexE	For	5′-TCATCCCACTTCTCCTGGCGCTACC-3′	150	This study
	Rev	5′-CGTCCCACTCGTTCAGCGGTTGTTCGATG-3′		This study
mexX	For	5′-AATCGAGGGACACCCATGCACATCC-3′	82	This study
	Rev	5′-CCCAGCAGGAATAGGGCGACCAG-3′		This study
Regulatory gene sequencing				
mexR	For	5′-TGTTCTTAAATATCCTCAAGCGG-3′	729	2
	Rev	5′-GTTGCATAGCGTTGTCCTCA-3′		2
	Int1	5′-GCGCAACCCCAGCGACCA-3′		This study
nalC	For	5′-TCAACCCTAACGAGAAACGCT-3′	813	2
	Rev	5′-TCCACCTCACCGAACTGC-3′		2
	Int 1	5′-CCGGCGATCGGCAAGTCC-3′		This study
	Int 2	5′-GATCCCCGCTGCCAGAGCC-3′		This study
nalD	For	5′-GCGGCTAAAATCGGTACACT-3′	788	2
	Rev	5′-ACGTCCAGGTGGATCTTGG-3′		2

ampC was associated with resistance to cephalosporins and piperacillin-tazobactam but not to carbenicillin or imipenem. Among β-lactams, only carbapenem resistance was associated with reduced expression of *oprD*. However, there were anomalies, including (i) an association of OprD deficiency with aminoglycoside resistance and (ii) no association between upregulation of efflux components and ciprofloxacin resistance.

Multivariate analysis (results not shown) identified increased *mexA* expression as the main mechanism of resistance to aztreonam ($P = 0.015$), carbenicillin ($P = 0.022$), and piperacillin-tazobactam ($P = 0.023$), and also to meropenem but only when resistance was defined biologically as a MIC >4 times the mode for the species (i.e., MIC of >2 μg/ml; $P = 0.027$), not at the clinical breakpoint ($P = 0.196$). Imipenem was not included in the multivariate analysis, because downregulation of *oprD* was the sole factor associated with resistance by univariate analysis. Surprisingly, no associations with upregulated *ampC* remained significant in the multivariate analysis, and no mechanisms remained associated with aminoglycoside resistance.

Mutations in at least three genes (*mexR*, *nalC*, and *nalD*) reportedly lead to increased expression of MexAB-OprM (4, 7, 18, 24, 26). We found numerous mutations in these genes in both clinical isolates and controls (Table 3), but no individual lesion was overrepresented among isolates with upregulated *mexA*; rather, 17 different combinations of *mexR*, *nalC*, and *nalD* mutations were seen. MexR Arg83Cys, NalC Gly71Glu, and NalD Asp187His were found in 11 of 14 representatives of the Liverpool strain, but these varied widely in resistance, including an up to 40-fold change in *mexA* expression. Arg83Cys in MexR is known to have an impact on efflux activity (25), whereas Gly71Glu in NalC is considered insignificant (18); to the best of our knowledge, Asp187His in NalD has not been reported before. The significance of this combination for *mexA* expression merits further investigation.

To conclude, we used RT-PCR to investigate complex combinations of resistance mechanisms in CF patient isolates of *P. aeruginosa*, many of them multiresistant. Despite many predicted statistical agreements between phenotype and gene up- or downregulation, the approach had limitations for analyzing resistance at the level of individual strains, with numerous anomalies, and showed some spurious statistical associations. Aside from distortions arising through multiple coresident mechanisms in the same strain, there were the following issues: (i) assay reproducibility, although use of HotStarTaq Plus DNA polymerase and assessment of expression using three separate RNA extractions resulted in values for all strains that clustered tightly around the means; (ii) RT-PCR provided only a snapshot of gene expression, which may vary through the growth cycle; (iii) *P. aeruginosa* has up to 13 efflux pump systems, and only the 4 best-characterized were studied here; (iv) the function of RND efflux pumps may be modulated not only by expression of the pump protein, as examined, but also by that of other components and by the architecture and energetics of the membrane within which it functions. Failure to associate resistance with an expected codeterminant, such as (i) between efflux-component overexpression and ciprofloxacin and (ii) only a weak association between overexpression of *mexX* and aminoglycoside resistance, suggests that other uninvestigated factors—mutations in DNA gyrase or topoisomerase IV and transmembrane aminoglycoside uptake or aminoglycoside-modifying enzymes, respectively—may be relatively more important than efflux as codeterminants of resistance. A more definitive evaluation would require isogenic strains, each

TABLE 2. Susceptibility and mRNA expression among control strains and clinical isolates of *P. aeruginosa*

Isolate	MIC (μg/ml)[a]											Relative gene expression[b]					
	IPM	MEM	PTZ	CAZ	CTX	ATM	CIP	CAR	GEN	TOB	AMK	*oprD**	*ampC*	*mexA*	*mexC*	*mexE*	*mexX*
Controls																	
M1251	4	8	32	4	128	32	0.5	512	0.5	0.25	1	**0.29**	1.78	**7.28**	1.24	**2.50**	**2.70**
R70	16	>32	32	8	256	64	0.5	>512	1	0.5	1	**0.05**	**5.72**	**5.94**	1.72	1.32	**7.94**
R20	2	0.5	8	1	4	4	≤0.12	64	≤0.25	0.5	≤0.5	**3.27**	0.73	0.75	0.77	**0.30**	1.79
AHP	0.12	0.25	4	0.5	4	0.5	≤0.12	32	≤0.12	≤0.12	≤0.5	1.23	**0.004**	**0.12**	**0.02**	**0.01**	**0.02**
Z799/61	0.5	≤0.06	≤1	≤0.12	0.5	≤0.12	≤0.12	≤16	≤0.12	0.25	1	**0.15**	**0.12**	**0.12**	**0.24**	**0.01**	**0.08**
2779-con	2	1	>64	32	>256	32	≤0.12	128	0.5	1	1	**5.34**	**313**	1.56	0.99	**4.90**	**3.71**
1405-con D2$^-$	16	8	>64	64	>256	64	0.25	512	2	2	4	**0.35**	**599**	**6.92**	**2.62**	**2.26**	1.22
Clinical isolates																	
1530[c]	8	0.5	≤1	4	32	2	8	64	32	4	32	**0.06**	1.13	0.65	0.69	1.23	**11.6**
1693[c]	8	2	≤1	8	128	1	1	128	16	2	1	**0.08**	**0.42**	**2.60**	**0.38**	**0.24**	**0.29**
17145[c]	32	4	2	64	128	0.5	4	≤16	32	16	64	**0.0004**	**8.40**	**0.39**	**0.11**	**0.33**	**2.55**
1529[c]	16	4	2	4	32	0.5	8	8	>32	16	64	**0.12**	**3.29**	0.56	**0.18**	1.18	**12.4**
14169[c]	16	4	8	8	128	64	1	512	32	4	32	**0.07**	**35.9**	**2.52**	1.05	**0.42**	**4.39**
1247[c]	16	4	4	8	128	4	4	128	>32	8	>64	**0.018**	**36.4**	1.81	0.75	**0.44**	**2.96**
1681[c]	32	8	>64	256	>256	32	2	512	32	16	64	**0.006**	**11.5**	**4.90**	**0.31**	**0.34**	**5.85**
1352[c]	>32	16	64	64	>256	32	2	512	>32	16	>64	**0.004**	**10.8**	1.82	**0.49**	0.89	**6.41**
1333[c]	32	16	>64	128	>256	>64	2	>512	>32	8	64	**0.0007**	**368**	**2.66**	1.77	**9.01**	**33.9**
1248[c]	16	16	32	256	>256	>64	4	>512	>32	32	>64	**0.012**	**5.89**	**4.11**	0.70	1.31	**11.7**
15110[c]	>32	16	>64	256	>256	>64	2	>512	>32	>32	>64	**0.05**	**474**	**5.61**	1.00	0.7	**2.37**
12142[c]	32	32	>64	256	>256	>64	4	>512	>32	>32	>64	**0.0004**	**262**	**15.9**	**3.1**	**3.7**	**6.52**
1712[c]	32	32	>64	>256	>256	>64	4	>512	>32	16	>64	**0.015**	**160**	1.83	1.2	**2.98**	**18.9**
1495[c]	>32	>32	>64	>256	>256	>64	4	>512	>64	8	>64	**0.06**	**284**	**3.49**	**2.92**	**6.91**	**78.3**
1726	4	0.12	≤1	2	16	0.5	2	≤16	2	0.5	2	**0.25**	1.50	1.08	**0.21**	**0.10**	1.80
15107	8	2	32	8	256	2	0.25	128	>32	2	>64	**0.03**	1.36	1.87	1.06	**0.26**	**10.5**
16008	64	4	≤1	1	64	0.5	4	≤16	16	4	32	**0.003**	**0.09**	0.56	**0.09**	**0.03**	**27.1**
13167	4	4	4	64	>256	16	8	>512	32	4	64	1.53	**7.22**	**3.51**	0.76	**0.4**	1.17
1240	16	8	8	256	256	4	2	128	2	0.5	4	**0.08**	**110**	1.30	1	1.82	**2.10**
3149	32	8	16	8	64	64	>8	>512	>32	>32	>64	**0.007**	**10.5**	**4.87**	1.35	**0.37**	**0.3**
12102	32	16	16	4	64	16	2	256	4	1	4	**0.0006**	0.79	**2.46**	1.51	1.02	**11.8**
1253	>32	32	64	8	128	16	2	256	16	4	32	**0.008**	**4.17**	1.96	**5.2**	**7.8**	**13.3**
13120	16	32	16	4	128	16	0.5	256	0.5	0.25	1	0.9	**2.04**	**2.30**	0.87	**0.4**	**15.0**
1665	64	>32	>64	256	>256	>64	4	>512	16	32	>64	**0.0001**	**150**	**5.89**	1.65	1.14	0.51
17112	128	>32	64	64	>256	>64	2	512	1	0.5	1	**0.03**	**44.1**	**3.12**	**0.17**	**0.08**	**0.11**

[a] IPM, imipenem; MEM, meropenem; PTZ, piperacillin-tazobactam; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; CIP, ciprofloxacin; CAR, carbenicillin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin.

[b] Increased or decreased gene expression relative to *P. aeruginosa* PAO1 (which was assigned a value of 1.0) was defined by expression values of ≥2 and ≤0.5, respectively, and is indicated in bold.

[c] Isolate belonging to the Liverpool epidemic strain.

TABLE 3. Combinations of mutations in genes reported to affect regulation of *mexA* expression

Mutation(s) detected			No. of isolates with mutation	
mexR	*nalC*	*nalD*	Clinical isolates	Control strains
Arg83→Cys	Gly71→Glu	Asp187→His	11[a]	—[b]
Arg83→Cys	Gly71→Glu	Asp187→His/Leu201→Pro	2	—
Arg83→Cys	Gly71→Glu	None	1	—
Arg83→Cys	Gly71→Glu/Ser209→Arg	Asp187→His/Leu201→Pro	1	—
Val126→Glu	Gly71→Glu	Asp187→His	1	—
Val126→Glu	Gly71→Glu	None	1	1
Val126→Glu	Gly71→Glu/Ser209→Arg	Ala145→Thr	—	1
Val126→Glu	Gly71→Glu/Ser209→Arg	None	—	1
Val126→Glu	Gly71→Glu/Ala186→Thr	Asp187→His	1	—
Val126→Glu	Gly71→Glu/Ser209→Arg/Ala78→Thr	None	—	1
None	Gly71→Glu	Asp187→His/Leu201→Pro	1	—
None	Gly71→Glu	None	3	—
None	Gly71→Glu/Ser209→Arg	Thr188→Ala	1	—
None	Gly71→Glu/Ser209→Arg	None	—	1
None	Gly71→Glu/Ala186→Thr	None	—	1
None	Gly71→Glu/Ser209→Arg/Ala145→Val	None	1	—
None	None	None	1	1

[a] All isolates were representatives of the Liverpool epidemic strain.
[b] —, no isolate.

differing by defined resistance mechanisms, and would include isolates with specific genes knocked out.

M. Tomás was financially supported by Program Río Hortega (CH Universitario A Coruña and ISCIII) and received a scholarship from the Spanish Society of Infections Diseases and Clinical Microbiology to stay in London. This work was partially funded by the Spanish Network for Research in Infectious Diseases (REIPI RD06/0008).

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