ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2006, p. 4174–4176 Vol. 50, No. 12
0066-4804/06/$08.00+0 doi:10.1128/AAC.00759-06

Resistance Mechanisms in an In Vitro-Selected Amoxicillin-Resistant Strain of *Helicobacter pylori*[▽]

Edgie-Mark A. Co and Neal L. Schiller*

Biomedical Sciences Division, University of California, Riverside, Riverside, California 92521

Received 22 June 2006/Returned for modification 25 August 2006/Accepted 14 September 2006

We investigated the β-lactam resistance mechanism(s) of an in vitro-selected amoxicillin-resistant *Helicobacter pylori* strain (AmoxR). Our results demonstrated that resistance is due to a combination of amino acid substitutions in penicillin binding protein 1 (PBP1), HopB, and HopC identified in AmoxR, resulting in decreased affinity of PBP1 for amoxicillin and decreased accumulation of penicillin.

Helicobacter pylori is the most common cause of gastric and duodenal ulcers and is strongly associated with the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (4). Treatment of *H. pylori* infection typically employs a triple-drug regimen using two antibiotics and a proton pump inhibitor or bismuth (4). However, resistance to the most commonly used antibiotics has already been observed worldwide (3, 8).

In a previous study (1), we isolated an amoxicillin-resistant mutant strain of *H. pylori* 43579 (designated AmoxR) with a MIC of 4 to 8 μg/ml by progressively culturing an amoxicillin-susceptible strain (AmoxS; MIC, 0.03 to 0.06 μg/ml) in increasing amounts of amoxicillin. The penicillin binding protein (PBP) profile of this strain showed a decreased affinity of PBP1 for amoxicillin, and AmoxR demonstrated a decrease in ^{14}C-labeled penicillin G accumulation (1). In this study, we report the presence of *pbp1* and *hop* mutations in the in vitro-selected amoxicillin-resistant mutant AmoxR and determine their effects on amoxicillin susceptibility.

The *pbp1* genes from AmoxS and AmoxR were amplified by PCR using primer sequences derived from the published 26695 sequence (12). The PCR products were sequenced at the University of California (UC), Riverside, Genomics Institute, and sequence alignment was performed in Sequencher (Gene Codes Corporation, Ann Arbor, MI). Comparison of the two *pbp1* sequences revealed a threonine-to-methionine conversion in the 438th amino acid position of PBP1 in AmoxR, located adjacent to an SLN motif, one of the penicillin binding domains (7). Others have also identified mutations localized around the SKN, SNN, and KTG motifs (7), which may be involved in β-lactam resistance (6, 9, 10, 11). To characterize the effect of this single T438M mutation in PBP1 on amoxicillin resistance, the gene was introduced into the well-characterized amoxicillin-susceptible (MIC, 0.06 μg/ml) *H. pylori* strain 700392, using electroporation as described by Wang et al. (13). Transformant selection was performed by plating electroporated cells onto brucella agar plates containing 5% sheep blood and amoxicillin at various concentrations. Several transformants (including PBP1-T) were randomly selected and found to have MICs for amoxicillin of 0.5 μg/ml, an eightfold increase compared to the parental strain (Table 1). Amplification and sequencing of the *pbp1* gene from PBP1-T confirmed that

TABLE 1. MICs of each β-lactam antibiotic for *H. pylori* 700392 and various transformants[a]

Strain	MIC						
	Amoxicillin	Ampicillin	Penicillin G	Ceftriaxone	Cefuroxime	Mezlocillin	Characteristics
700392	0.06	0.06	0.06	0.5	0.5	1	ATCC type strain
PBP1-T	0.5	0.5	0.5	0.5	1	1	PBP1 transformant
HopB-T	0.25	0.5	1	1	1	2	HopB transformant
HopC-T	0.125	0.5	0.5	1	0.5	1	HopC transformant
PBP1-T/HopB-T	2	4	2	4	2	4	PBP1/HopB double mutant
PBP1-T/HopC-T	1	2	1	2	1	2	PBP1/HopC double mutant
PBP1/HopB/HopC triple mutant	4	8	2	2	2	4	Triple mutant
AmoxR	4–8	4–8	4–8	4–8	4–8	2–4	In vitro-selected strain

[a] MICs were determined after 48 h of incubation at 37°C by a broth microdilution method as described previously (1). The data shown (in μg/ml) are the modes of at least three separate experiments.

* Corresponding author. Mailing address: Biomedical Sciences Division, University of California, Riverside, Riverside, CA 92521-0121. Phone: (951) 827-4535. Fax: (951) 827-5504. E-mail: neal.schiller@ucr.edu.

▽ Published ahead of print on 25 September 2006.

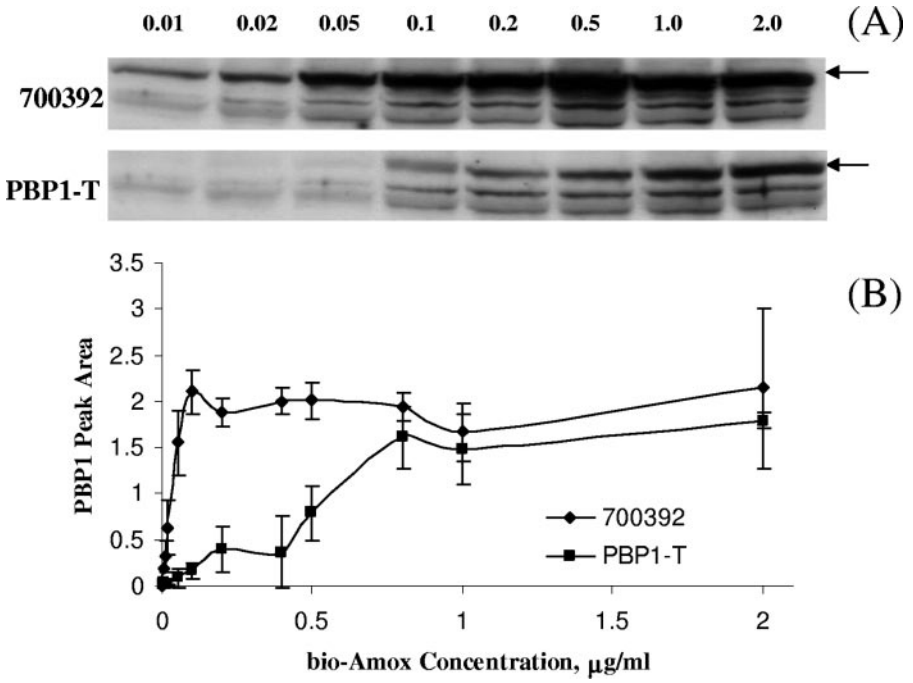

FIG. 1. (A) Bio-Amox labeling of 700392 and PBP1-T PBPs. Equivalent amounts of membrane proteins were exposed to increasing amounts of bio-Amox to saturation. The numbers indicate bio-Amox concentrations (μg/ml) used to label PBP1. The arrows indicate the locations of the PBP1 band; the two lower bands correspond to PBP2 and PBP3. (B) Quantitative densitometric tracings of PBP1 bands. The data represent the means ± standard deviations based on four separate experiments (including the gel shown in panel A).

the transformant possessed the mutant *pbp1* from AmoxR. PBP1-T also had comparably increased MICs for ampicillin and penicillin G, although little or no increase in MIC levels was noted for the other β-lactams examined, suggesting that this particular *pbp1* mutation in AmoxR affects the binding affinity of PBP1 for only a few specific antibiotics.

To compare the affinity of PBP1 for amoxicillin in 700392 and PBP1-T, we titrated the binding of PBP1 using biotinylated amoxicillin (bio-Amox) as described previously (1). In this study, equivalent amounts of membrane proteins were labeled for 10 min at room temperature with bio-Amox concentrations ranging from 0.001 to 2 μg/ml. At very low to moderate amoxicillin concentrations, the PBP1 in PBP1-T bound substantially less amoxicillin than the PBP1 in the susceptible parental strain (Fig. 1); it required more than eight times as much bio-Amox to achieve equivalent binding of PBP1 in PBP1-T as in 700392. Interestingly, the saturation of PBP1 with bio-Amox in both strains occurred at concentrations near their respective MICs (Fig. 1B). For strain 700392, saturation binding started between 0.05 and 0.1 μg/ml (MIC = 0.06), while in the transformant PBP1-T, saturation binding occurred at 0.8 μg/ml (MIC = 0.5). These data suggest a possible correlation between the

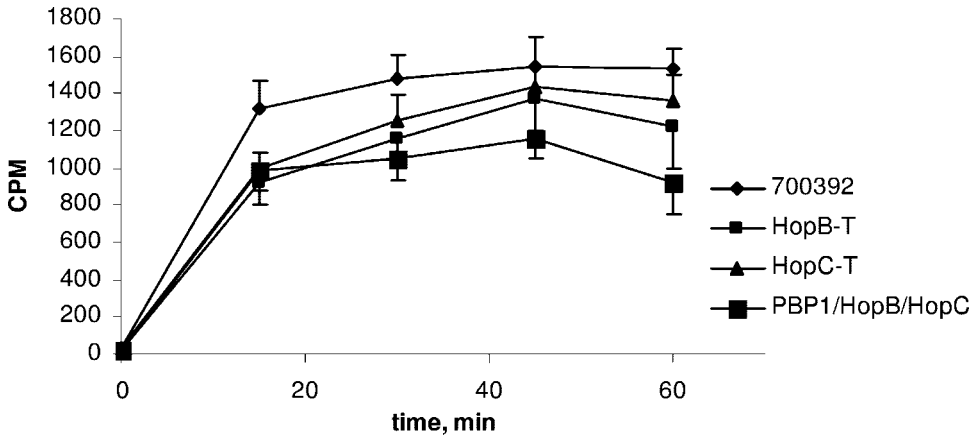

FIG. 2. ^{14}C-labeled penicillin G accumulation of *H. pylori* 700392 and *hop* transformants. Log-phase cultures were incubated with ^{14}C-labeled penicillin G, and 1-ml aliquots were taken at 15, 30, 45, and 60 minutes, centrifuged, and washed, and radioactivity was measured in a scintillation counter. The error bars represent the means ± standard errors of the means based on three separate experiments.

degree of amoxicillin binding and the level of susceptibility to amoxicillin.

In our previous study, AmoxR accumulated >40% less ^{14}C-labeled penicillin G than equal numbers of AmoxS bacteria (1). Since decreased permeability of antibiotics through the outer membrane of AmoxR could account for the lower accumulation of ^{14}C-labeled penicillin G, we focused on *H. pylori* porin proteins (2, 5, 12). Sequence alignments of the genes *hopA*, *hopD*, and *hopE* from AmoxR, compared to their parental counterparts in AmoxS, did not reveal any amino acid substitutions. On the other hand, the *hopB* gene of AmoxR possesses a number of mutations localized in the amino acid 116 to 201 region, and the *hopC* gene possesses a stop codon at the 211th amino acid position (data not shown). When these genes were transformed into 700392, the resulting strains were observed to have increased MICs for amoxicillin of 0.25 μg/ml for HopB-T and 0.125 μg/ml for HopC-T (Table 1). When the MICs for other antibiotics were tested, both transformants had elevated MICs for ampicillin and penicillin G but little or no increase for the other β-lactams tested.

Subsequent experiments revealed decreased ^{14}C-labeled penicillin G accumulation in the *hop* mutants, approximately 20% and 10% less than that in the parental strain for HopB-T and HopC-T, respectively, after 60 min of incubation. The most dramatic decrease was observed at the 15-min time point, when ~30% less ^{14}C-labeled penicillin G had accumulated in both strains (Fig. 2).

Double mutants possessing *pbp1*-T438M and altered *hop* genes were constructed and showed increased MICs for all β-lactams tested in an additive fashion, particularly the PBP1-T/HopB mutant (Table 1), indicating that the PBP1 and HopB alterations work in concert to decrease the effectiveness of the antibiotics. When both *hop* genes were transformed into PBP1-T, creating a triple mutant, a further decrease in ^{14}C-labeled penicillin G accumulation (Fig. 2), as well as a concurrent increase in the MIC, was observed (Table 1). The MIC level for amoxicillin observed in the triple mutant was 4 μg/ml, comparable to that seen in AmoxR (4 to 8 μg/ml). In addition, the ^{14}C-labeled penicillin G accumulation levels in this triple mutant reflected about a 40% decrease compared to the parental strain at the 60-min time point (Fig. 2), comparable to that observed with AmoxR (1). These experiments suggest that the amino acid substitutions affecting PBP1, HopB, and HopC account for all the resistance mechanisms found in AmoxR.

This research was supported in part by grants from UC MEXUS and the UC Cancer Research Coordinating Committee. E.-M. A. Co acknowledges support provided by a Graduate Division Dissertation Grant at UCR.

REFERENCES

1. **DeLoney, C. R., and N. L. Schiller.** 2000. Characterization of an in vitro-selected amoxicillin-resistant strain of *Helicobacter pylori*. Antimicrob. Agents Chemother. **44:**3368–3373.
2. **Doig, P., M. M. Exner, R. E. W. Hancock, and T. J. Trust.** 1995. Isolation and characterization of a conserved porin protein from *Helicobacter pylori*. J. Bacteriol. **177:**5447–5452.
3. **Duck, W. M., J. Sobel, J. M. Pruckler, Q. Song, D. Swedlow, C. Friedman, A. Sulka, B. Swaminathan, T. Taylor, M. Hoekstra, P. Griffin, D. Smoot, R. Peek, D. C. Metz, P. B. Bloom, S. Goldschmid, J. Parsonnet, G. Triadafilopoulos, G. I. Perez-Perez, N. Vakil, P. Ernst, S. Czinn, D. Dunne, and B. D. Gold.** 2004. Antimicrobial resistance incidence and risk factors among *Helicobacter pylori*-infected persons, United States. Emerg. Infect. Dis. **10:**1088–1094.
4. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. **10:**720–741.
5. **Exner, M. M., P. Doig, T. J. Trust, and R. E. W. Hancock.** 1995. Isolation and characterization of a family of porin proteins from *Helicobacter pylori*. Infect. Immun. **63:**1567–1572.
6. **Gerrits, M. M., D. Schuijffel, A. A. van Zwet, E. J. Kuipers, C. M. J. E. Vanderbrouke-Grauls, and J. G. Kursters.** 2002. Alterations in penicillin-binding protein 1A confer resistance to β-lactam antibiotics in *Helicobacter pylori*. Antimicrob. Agents Chemother. **46:**2229–2233.
7. **Harris, A. G., S. L. Hazell, and A. G. Netting.** 2000. Use of digoxigenin-labelled ampicillin in the identification of penicillin-binding proteins in *Helicobacter pylori*. J. Antimicrob. Chemother. **45:**591–598.
8. **Kursters, J. G., and E. J. Kuipers.** 2001. Antibiotic resistance of *Helicobacter pylori*. J. Appl. Microbiol. **90:**134s–144s.
9. **Kwon, D. H., M. P. Dore, J. J. Kim, M. Kato, M. Lee, J. Y. Wu, and D. Y. Graham.** 2003. High-level β-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. **47:** 2169–2178.
10. **Okamoto, T., H. Yoshiyama, T. Nakazawa, I.-D. Park, M.-W. Chang, H. Yanai, K. Okita, and M. Shirai.** 2002. A change in PBP1 is involved in amoxicillin resistance of clinical isolates of *Helicobacter pylori*. J. Antimicrob. Chemother. **50:**849–856.
11. **Paul, R., S. Postius, K. Melchers, and K. P. Schafer.** 2001. Mutations of the *Helicobacter pylori* genes *rdxA* and *pbp1* cause resistance against metronidazole and amoxicillin. Antimicrob. Agents Chemother. **45:**962–965.
12. **Tomb, J.-F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quakenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzgerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:** 539–553.
13. **Wang, Y., K. P. Roos, and D. E. Taylor.** 1993. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J. Gen. Microbiol. **139:**2485–2493.