JOURNAL OF BACTERIOLOGY, Nov. 1980, p. 823-825
0021-9193/80/11-0823/03$02.00/0

Vol. 144, No. 2

NOTES

Tetracycline-Stimulated Expression of Ampicillin Resistance in *Haemophilus influenzae*

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Tetracycline at a low concentration stimulated the expression of ampicillin resistance in certain strains of *Haemophilus influenzae*.

Bryan (1) reported the isolation of an *Haemophilus influenzae* strain which carried a conjugative resistance plasmid with Amp^r, Cam^r, and Tet^r determinants (resistance against ampicillin, chloramphenicol, and tetracycline, respectively). When the strain was mated with a suitable recipient, transconjugant colonies appeared in agar with tetracycline but did not appear in agar with ampicillin; yet the Tet^r transconjugants were resistant to chloramphenicol and to ampicillin. They also carried a large plasmid. Experiments in this laboratory confirmed Bryan's observations. It was also demonstrated that the transfer of Tet^r by genetic transformation occurred readily, but that the transfer of Amp^r did not. However, all Tet^r transformants tested were resistant to chloramphenicol and to ampicillin. Although the Tet^r transformants did not possess a free large plasmid, they were subsequently shown to have an integrated conjugative R plasmid (4). Broth suspensions without ampicillin gave very low viable counts when the cells were plated in agar with ampicillin at 5 μg/ml, whereas the expected counts were obtained when the cells were plated in agar with tetracycline at 3 μg/ml. The Tet^r subclones were also resistant to chloramphenicol and ampicillin. Thus, it appeared that resistance to ampicillin often was not expressed fast enough for survival in cells carrying this triple resistance plasmid (Table 1).

Interestingly, when 0.1 μg of tetracycline per ml was added to the agar with ampicillin, predicted Amp^r viable counts were observed. Mating of the *H. influenzae* strain with a strain Rd recipient (3) resulted in similar numbers of Tet^r and Amp^r transconjugants when this ampicillin agar was used. Genetic transformation of Rd cells with DNA from the triply resistant strain gave the same frequency of Tet^r and Amp^r transfer (Table 1). These observations suggested that a trace of tetracycline might act as an inducer in an operon-type structure in which the Amp^r and Tet^r genes are linked. Alternatively, it was also possible that the sublethal amount of tetracycline acted in an indirect way by slowing down protein synthesis and cell division, thereby allowing these cells more time for the expression of ampicillin resistance.

To distinguish between these two possibilities, an Rd strain carrying the Amp^r plasmid pJS1842 (see references 3 and 4 for strain and plasmid details) was grown up in broth and plated in ampicillin agar at 6 μg/ml and in the same agar containing various protein synthesis inhibitors at about half the minimum lethal concentration. The plates were counted after overnight incubation at 37°C. Expected viable counts of about 2×10^9 per ml were observed in ampicillin agar with either tetracycline (0.1 μg/ml), oxytetracycline (0.1 μg/ml), chlortetracycline (0.05 μg/ml), chloramphenicol (0.15 μg/ml), spectinomycin (3 μg/ml), or oleandomycin (25 μg/ml). Much lower viable counts (by over 100 times) were seen in agar with ampicillin (6 μg/ml) or in this agar with either kanamycin (1 μg/ml), neomycin (2.5 μg/ml), paramomycin (2 μg/ml), gentamicin (0.15 μg/ml), or puromycin (1 μg/ml). The inhibitors alone did not reduce the viable counts, but they did slow down cell division since the colonies were much smaller. These observations thus rule out a tetracycline-specific induction for the expression of ampicillin resistance.

From a practical point of view, it was important to determine whether other *H. influenzae* strains would give this tetracycline stimulation effect. About 20,000 cells from overnight suspensions were plated (i) in plain agar, (ii) in agar with 6 μg of ampicillin per ml, and (iii) in the same ampicillin agar with 0.1 μg of tetracycline per ml. The results of two experiments showed a positive effect for strains NT1056, Sb1057, Sb1245, Sb1497, NT1759 (2), NT1760, NT1772, Sb1842, NT1863, Sb1864, and NT1897 (1). The

TABLE 1. *Tetracycline-stimulated expression of ampicillin resistance in H. influenzae*

Strain	Viable count (colony-forming units per ml) in agar with:			
	No antibiotic	Tetracycline[a]	Ampicillin[b]	Ampicillin[b] and tetracycline[c]
NT1897(pLEB1)	2.2×10^9	2.6×10^9	10^7	2.2×10^9
Transformants [DNA from NT1897(pLEB1)][d]		1.1×10^3	<10	8.9×10^2
Transconjugants				
Donor NT1897(pLEB1)[d]		7.5×10^2	<30	8.2×10^2
Donor Rd1898(pLEB1)[d, e]		1.0×10^5	6.0×10^2	1.2×10^5

[a] At 3 μg/ml.
[b] At 5 μg/ml.
[c] At 0.1 μg/ml.
[d] The recipient was strain Rd.
[e] Transconjugants resistant to ampicillin and tetracycline (3 μg/ml), 1.5×10^2; to ampicillin and chloramphenicol (3 μg/ml), 1.0×10^4; to chloramphenicol, 5.0×10^3; to chloramphenicol and tetracycline (3 μg/ml), 2.7×10^3; and to ampicillin, chloramphenicol, and tetracycline (3 μg/ml), 1.2×10^2.

following strains were not stimulated: NT1494, NT1495, Sb1498, Sb1729 (also Tet^r), Sb1735, and Sb1867. There seemed to be no correlation between stimulation and the state of the plasmid, as some of the strains carried a free plasmid whereas others had integrated plasmids (4).

The influence of the ampicillin concentration in the agar was demonstrated with strains Rd1318(pJS1245) (integrated) and Sb1497-(pJS1497) (integrated). Cells from plain broth were plated in plain agar or in agar containing 3, 6, or 9 μg of ampicillin per ml, with and without 0.1 μg of tetracycline per ml. Expected viable counts were seen in agar with 3 μg of ampicillin per ml independent of added tetracycline and in ampicillin agar at 6 and 9 μg/ml with tetracycline. Tetracycline-stimulated expression of ampicillin resistance thus depended on the ampicillin concentration used in the agar. It is possible that those strains that were not stimulated may be stimulated if examined at higher ampicillin concentrations.

To determine whether a trace of tetracycline in broth would stimulate ampicillin resistance, strains Rd1318(pJS1245) (integrated) and Rd1900(pLEB1) (integrated) were cultured twice in plain broth and in broth with 0.1 μg of tetracycline per ml. The four suspensions were plated in ampicillin agar at 6 μg/ml with and without 0.1 μg of tetracycline per ml. Predicted viable counts for all four suspensions were obtained only in the agar which contained both ampicillin and tetracycline. Sublethal amounts of tetracycline in broth thus do not influence the expression of ampicillin resistance when the cells are plated in ampicillin agar.

Since ampicillin resistance of resistant *H. influenzae* is due to the production of β-lactamase, it was of interest to determine whether such strains produce this enzyme in the absence of ampicillin or tetracycline or both. The following strains were streaked on plain agar and on agar with either ampicillin at 3 μg/ml or tetracycline at 0.1 μg/ml: Sb1057(pJS1057-2), Sb-1245(pJS1245), and Sb1864(pJS1864) (all integrated); NT1760(pJS1760), NT1772(pJS1772), and NT1897(pLEB1) (all free); NT1862 (plasmid status not determined); and Rd1287 and Rd1292 (both sensitive to ampicillin; control cultures). All plates were incubated overnight at 37°C. One drop (about 0.05 ml) of β-lactamase reagent (1.0 ml of 0.4% soluble starch in distilled water, 0.4 ml of 1.5% KI–0.3% iodine in 0.1 M phosphate buffer at pH 6.4, 0.2 ml of a 0.5-g/ml penicillin solution) was placed on each streak. The dark violet color of the reagent faded within 30 s on all streaks of the resistant cultures, whereas it took more than 5 min to do so on the control streaks. Thus, all resistant cultures produced β-lactamase, even on agar without ampicillin.

Overnight suspensions of the above strains in plain broth and in broth containing ampicillin at 3 μg/ml or tetracycline at 0.1 μg/ml were mixed at a 1:1 ratio with the β-lactamase reagent. The resistant cultures gave positive scores under all conditions, whereas the control suspensions gave only very slow fading of the violet color.

These observations suggest that the stimulation of ampicillin resistance in agar with a trace of tetracycline is caused by the slowing down of cell growth through partial inhibition of protein synthesis, thereby making the cells less sensitive to ampicillin-mediated inactivation. It is not clear why some strains did not shown this stimulation effect or why some protein synthesis inhibitors were inactive. However, to study the loss of ampicillin resistance plasmids, it may be

productive to examine the strains for this stimulation effect.

I am grateful to L. E. Bryan for sending me the *H. influenzae* isolate with the pLEB1 plasmid.

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