New Immunoenzymatic Strategy for Rapid and Selective Growth of *Salmonella*

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We demonstrated that an exogenous conjugate consisting of a β -lactamase and a monoclonal antibody against a surface antigen of *Salmonella* is capable of conferring selective penicillin resistance on bacteria. This immunoenzymatic approach allows the rapid growth and detection of specific bacteria and may find other applications when temporary and nongenetic antibiotic resistance is required.

In bacterial detection methods, pre-enrichment is a preliminary step that is generally required to provide a sufficient amount of material for a subsequent microbiological, immunological, or PCR-based assay. Selective media are used for this purpose, but their stringent composition results in slow growth. In a medical or food-processing environment, minimization of the time required is of prime importance but efforts have been concentrated on the improvement of assay sensitivity and very few methods are developed for speeding up the time-consuming pre-enrichment step. The latest technological improvement is the use of immunomagnetic separation for extraction of the target bacteria from a food sample (4), but such an additional manipulation complicates the protocol and is not always easy to implement with complex samples. In this study, we simply brought selectivity to a rich nonselective medium by the use of two additives, a β -lactam antibiotic that kills most of the bacteria in the sample and a β -lactamase-antibody conjugate that selectively protects the target bacteria (Fig. 1A). With *Salmonella* as a model pathogenic bacterium, our results unambiguously demonstrate that fastening exogenous β -lactamase to the surface of the bacteria is sufficient to protect them against high concentrations of ampicillin.

A commercial mouse monoclonal IgG antibody to *Salmonella enterica* serovar Typhimurium (C65336M; Meridian Life Sciences, Inc.) and the TEM-1 β -lactamase purified as a His-tagged protein by metal affinity chromatography were individually biotinylated. Solutions containing, respectively, 26.6 μ M TEM-1 β -lactamase and 2 μ M antibody were incubated overnight at 4°C with a 10-fold molar excess of Biotin-LC-NHS (Pierce) in NaHCO₃ buffer (0.1 M, pH 8.0) and then dialyzed against phosphate-buffered saline (PBS). These proteins were assembled into a conjugate by premixing in a PBS solution before the addition of streptavidin (gift from Thomas R. Ward). The antibody-streptavidin- β -lactamase (ASB) mixture was analyzed by nonreducing SDS-PAGE without boiling the sample to preserve the streptavidin-biotin assembly (Fig. 1B). The bands corresponding to both the antibody and β -lactamase progressively disappeared upon the addition of increasing concentrations of streptavidin, and high-molecular-mass (>150-kDa) complexes appeared. At a 1:1:1 molar ratio, all of the biotinylated proteins were conjugated with streptavidin. The rate of benzylpenicillin hydrolysis by the final 1:1:1 conjugate was measured by spectrometry at 232 nm [$\Delta\epsilon_{(S-P)} = 1,030 \text{ M}^{-1} \text{ cm}^{-1}$]. A final activity of 67.8 units/ml was calculated. The conjugate is a mixture of different compounds (A_xSB_y ,

with x and y between 0 and 4 and $x + y = \leq 4$) and was used as such.

The ASB conjugate was used as an additive together with ampicillin in a classical lysogeny broth (LB) supplemented with 1% bovine serum albumin (BSA) for growing *Salmonella* (ATCC 29631 strain). The quantity of ASB conjugate was adjusted to hydrolyze the ampicillin in the first hour of culture by using the previously measured activity and assuming that the enzyme is 11% more active on ampicillin than on benzylpenicillin (1). For each experiment, the medium was inoculated with bacteria and the ASB conjugate was then added and mixed. Five minutes later, the ampicillin was added at the desired concentration and growth at 37°C was monitored. As shown in Fig. 1C, *Salmonella* was fully protected by the ASB conjugate in the initial presence of 400 mg/liter of ampicillin since its growth rate was identical to the observed rate without ampicillin. Replacement of the ASB conjugate with β -lactamase alone did not provide any protection of the bacteria. Similar results were obtained by using a 3-fold increase in both the ampicillin and ASB conjugate concentrations. As an additional negative control, the ASB conjugate was unable to confer ampicillin resistance on a closely related bacterium such as *Escherichia coli*.

The detection and selective enrichment of *Salmonella* starting from a mixture of bacteria was then tested. Cultures of 20 ml inoculated with various numbers of CFU of *Salmonella* and *E. coli* (kanamycin-resistant DH5 α F' IQ strain; Invitrogen) were grown for 24 h at 37°C with or without addition of the ASB-ampicillin cocktail. The titers were calculated by plating serial dilutions on petri dishes with or without kanamycin (25 μ g/ml). As shown in Table 1, the cocktail afforded quasiabsolute selective growth of *Salmonella* starting from as few as about 10 CFU mixed with 1,000 CFU of *E. coli*. The ASB conjugate was also used as a detection tool with nitrocefin, a chromogenic substrate of β -lactamase that turns red upon hydrolysis. In an enzyme-linked immunosorbent assay (ELISA)-like experiment, 2-ml samples of culture harvested after

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FIG 1 (A) Immunoenzymatic principle of conferring specific penicillin resistance on a bacterium by adding an ASB conjugate to the culture broth. (B) Nonreducing SDS-PAGE analysis of the ASB conjugate. Samples were not heated. Lanes 1 to 3, respectively, biotinylated β -lactamase (25 pmol, 0.75 μ g), biotinylated antibody (25 pmol, 3.7 μ g), and streptavidin (40 pmol, 2.6 μ g). Lanes 4 to 6, mixtures containing 25 pmol of both biotinylated proteins and increasing amounts of streptavidin (respectively, 6.25, 12.5, and 25 pmol). Lane MW, molecular size markers. (C) Growth curve of *Salmonella* in LB broth supplemented with 1% BSA, ampicillin (400 μ g/ml), and a quantity of ASB capable of hydrolyzing the antibiotic in 1 h. Controls with ampicillin alone, with nonconjugated β -lactamase, and without the additives demonstrate the full protective effect of the conjugate. OD₆₀₀, optical density at 600 nm. (D) Detection of a few cells of *Salmonella* in a mixture with *E. coli* after 6 or 16 h of culture in the presence of the ASB-ampicillin cocktail. (E) Detection of a few cells of *Salmonella* in contaminated yogurt after 6 h of culture in the presence of the ASB-ampicillin cocktail.

6 and 16 h of selective culture were centrifuged at 14,000 rpm for 5 min. The pellet was resuspended in 100 μ l of phosphate buffer (100 mM, pH 7), 1 pmol of ASB conjugate (streptavidin equivalent) was added, and the mixture was incubated for 30 min. Bacteria were then centrifuged, resuspended in 1 ml of phosphate

buffer for washing, centrifuged again, and finally resuspended in 100 μ l of phosphate buffer containing nitrocefin (100 μ M). Images were taken when the best contrast between positive and negative controls was obtained (less than 20 min). As shown in Fig. 1D, a positive signal was detected after only 6 h of selective growth using 1/10 of a culture initially inoculated with around 10 CFU of *Salmonella* and 10^6 CFU of *E. coli*.

Finally, 2-ml liquid yogurt samples (Actimel; Danone) containing high titers of *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus casei* were artificially contaminated with around 10 CFU of *Salmonella* and diluted 10-fold in LB with or without the ASB-ampicillin cocktail. After 6 h of culture at 37°C, 2 ml was analyzed with the ELISA-like assay. Figure 1E shows that *Salmonella* was clearly detected when the cocktail was added. Without the cocktail, the competition between the bacteria is most probably slowing down the growth of *Salmonella*, thus preventing its detection after 6 h.

TABLE 1 Selective enrichment from *E. coli*-*Salmonella* mixed cultures

Bacterial inoculum (CFU)	Ampicillin + ASB conjugate ^a	% <i>Salmonella</i>	
		<i>t</i> = 0 h	<i>t</i> = 24 h
10^8	—	1	5
10^8	+	1	>99
10^7	—	18	17
10^7	+	18	>99
10^4	+	1	>99
10^3	+	1	98

^a —, absent; +, present.

Our work demonstrates that β -lactam resistance can be selectively provided to *Salmonella* by fastening a β -lactamase to its surface via a specific antibody. Using such a conjugate-antibiotic cocktail in a rich broth affords the rapid and selective growth of the target bacteria. The method is very simple and should find useful applications in medical and food-processing environment where the detection of pathogens must be as fast as possible. Using a single growth step, we were able to detect a few CFU of *Salmonella* in less than a working day whereas all of the commercial kits that we are aware of for the detection of *Salmonella* have protocols requiring at least 2 days because of the necessity of a more-or-less selective pre-enrichment step, followed by a rapid enrichment step.

With our method, any intrinsically ampicillin-resistant bacteria in the sample will also grow in the medium and potentially reduce the time required for ampicillin hydrolysis but, for the purpose of pre-enrichment, this may not be a problem if the titer of resistant bacteria is lower than the *Salmonella* titer. Using what we know about the β -lactamase content of a resistant *E. coli* strain transformed with pBR322, we estimated that we would need more than 10,000 resistant cells per ml to really affect the assay at 1,200 mg/ml ampicillin. A solution to that potential problem will be to use a conjugate with an extended-spectrum β -lactamase together with an expanded- or broad-spectrum β -lactam compound.

The method is also applicable whenever a temporary and non-genetic antibiotic resistance is required. For example, *Salmonella* is being developed as an anticancer vector (3) but the strains must be antibiotic sensitive because of biosafety regulations (2). It is, however, potentially interesting to be able to use antibiotics and the bacteria simultaneously to avoid or combat possible infections during treatment.

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